University of Alberta

The depressed hepatic retinyl ester hydrolase (REH) contributes to the impaired metabolic availability of vitamin A in BB rats

by

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List of Abbreviations

ALT	L-alanine: 2-oxoglutarate aminotransferse
ANOVA	Analysis of variance
AOAC	Association of analytical communities
ARAT	Acyl-CoA:retinol acyltransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBd rats	Diabetic bio-breeding rats
BBdp rats	Diabetes-prone bio-breeding rats
BBn rats	Non-diabetes-prone bio-breeding rats
cDNA	Complementary deoxyribonucleic acid
CEL	Carboxylester lipase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CI	Confidence interval
CRBP	Cellular retinol binding protein
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
$E^{1\%}_{1cm}$	Extinction Coefficient
EDTA	Ethylenediaminetetraacetic acid
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
HVA diet	High vitamin A diet
IDL	Intermediate density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LRAT	Lecithin:retinol acyltransferase
LVA diet	Low vitamin A diet
mRNA	Messenger ribonucleic acid
NDSS	National diabetes surveillance system
PBS	Phosphate-buffered saline

PTL	Pancreatic triglyceride lipase
RBP	Retinol binding protein
RE	Retinol equivalent
REH	Retinyl ester hydrolase
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
SOD	Superoxide dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triglyceride
TTR	Transthyretin
VLDL	Very low density lipoprotein

Chapter 1
Introduction

1.1 Overview

Diabetes mellitus is a clinically and genetically heterogeneous group of chronic metabolic disorders characterized by abnormally increased level of glucose in the blood (American Diabetes Association 2004). This hyperglycemia results from an inability of the body to sufficiently produce or properly use insulin, or a combination of both. Diabetes mellitus poses a serious and growing public health problem both in Canada and worldwide. According to the National Diabetes Surveillance System (NDSS) of Canada, 5.1% of Canadians aged 20 and over, or 1.2 million adults in Canada, were diagnosed as diabetes in 1999/2000 (National Diabetes Surveillance System 2004). This number is expected to reach 3 million by 2010 (Meltzer et al. 1998). The chronic hyperglycemia of diabetes is often associated with long-term damage, dysfunction, and failure of various organs, including the eyes, kidneys, nerves, heart and blood vessels (Skyler 2001). Diabetes mellitus per se is the 6th leading cause of death in Canada and it is closely related to cardiovascular disease, which is the number one cause of death in this country (Meltzer et al. 1998;Cardiovascular Disease Surveillance 2004). Diabetes mellitus also commonly leads to end-stage renal disease, new onset blindness, and lower limb amputations in adults (Åkerblom and Knip 1998). The cost of diabetes in Canada is estimated to be nearly $9 billion annually (Health Canada 2004). With the aging of our population, the burden of diabetes and its complications will become increasingly prevalent and costly in the future.

Diabetes mellitus is commonly associated with abnormalities in carbohydrate, fat, and protein metabolism (American Diabetes Association 2004). The mechanisms by which these metabolic disturbances occur in diabetes are fairly well understood. The relative or absolute deficiency of insulin, as well as elevated counter-regulatory hormones (such as glucagons, growth hormone, and catecholamines) in diabetes lead to excessive hepatic release of glucose and impaired muscle glucose uptake, which in turn cause the high levels of plasma glucose in diabetic subjects (Dinneen et al. 1992). The defects in insulin secretion and action also activate hormone-sensitive lipase in adipose tissue and increase the plasma free fatty acid concentrations (Durrington 1999). In addidition, insulin has been shown to regulate enzymes such as adenosine triphosphate (ATP)-independent lysosomal proteases and hydrolases, which are responsible for tissue protein breakdown. Lack of insulin in diabetes thus stimulates protein breakdown and further increases amino acid oxidation in diabetic patients (Abu-Lebdeh and Nair 1996).

In contrast to the well established and explained metabolic derangement of carbohydrate, fat and protein in diabetes, the metabolic abnormalities of micronutrients under the diabetic state have been poorly understood. Various studies examined the effect of diabetes on the metabolism and concentrations of some vitamins and minerals, including vitamin C (Birlouez-Aragon et al. 2001;Will et al. 1999;Sinclair et al. 1994), vitamin E (Cinaz et al. 1999;Campoy et al. 2003), vitamin D (Ishida et al. 1985;Nyomba et al. 1986), zinc (Anetor et al. 2002;McNair et al. 1981;Isbir et al. 1994), chromium (Rukgauer and Zeyfang 2002;Wells et al. 2003), and magnesium (Engelen et al. 2000a;Anetor et al. 2002;Takaya et al. 2003). Clinical trials have been conducted to determine the effect of dietary supplementation of some of these micronutrients in diabetic patients (Darko et al. 2002;Engelen et al. 2000b;Morris et al. 2000;Lal et al. 2003;Roussel et al. 2003). Most results from these studies, however, remain inconclusive as well as controversial.

In the past decade, patients with diabetes mellitus have been linked to an increased risk of marginal vitamin A deficiency. Human studies showed that plasma vitamin A levels were decreased in diabetic patients (Basu et al. 1989;Krempf et al. 1991;Martinoli et al. 1993;Krill et al. 1997;Merzouk et al. 2003;Baena et al. 2002). This reduced plasma vitamin A level is often observed in type 1 but not type 2 diabetic patients (Basualdo et al. 1997;Abahusain et al. 1999).

The decreased vitamin A status in type 1 diabetes is of clinical significance since 1) vitamin A deficiency may further affect the secretion of insulin (Chertow et al. 1987;Chertow et al. 1989); and 2) both diabetes and vitamin A deficiency may affect vision (Fong et al. 2004;Smith and Steinemann 2000). Statistics have shown that diabetic retinopathy is the leading cause of new cases of legal blindness among Americans between the ages of 20 to 74 y (Fong et al. 2004). In type 1 diabetes of 15 or more years' duration, the risk of retinopathy can be up to 98% (Davis 1992). Although the mechanism for blindness is different in diabetes and in vitamin A deficiency, it is conceivable that retinopathy in diabetes can be further exacerbated by the presence of a marginal vitamin A deficiency. It is therefore important to acquire a better understanding of the depressed vitamin A status in type 1 diabetes.

The cause(s) of depressed vitamin A status in type 1 diabetes is not yet clarified. Results from several animal studies in both chemically-induced and spontaneously developed type 1 diabetic model (rats) show that, in contrast to the decreased plasma retinol level, hepatic total vitamin A concentration is similar (Lu et al. 2000) or even higher (Tuitoek et al. 1996c) in diabetic rats compared with non-diabetic controls. The plasma or hepatic retinol binding protein (RBP) concentrations and hepatic RBP messenger ribonucleic acid (mRNA) expression are decreased in diabetic rats (Tuitoek et al. 1996b;Lu et al. 2000). These results suggest that the transport of retinol from the liver to the circulation is depressed in the diabetic state, and this reduced transport of retinol is probably induced by decreased synthesis and/or secretion of RBP in the liver. Results from these studies also suggest that disturbed zinc status in type 1 diabetes might play an important role in reduced RBP synthesis and secretion.

Retinyl ester hydrolysis is a key step involved in the intestinal uptake of dietary vitamin A, hepatic uptake of chylomicron remnant-associated retinyl ester as well as the hepatic mobilization of retinyl esters from the vitamin A storage pools. This process thus plays critical roles in vitamin A homeostasis in the body. The main objectives of this thesis are therefore to examine if alterations in retinyl ester hydrolysis contributes to the depressed vitamin A status and how dietary modifications of vitamin A and zinc affect retinyl ester hydrolysis in a diabetic state.

1.2 Vitamin A metabolism

Vitamin A is an essential nutrient that must be provided in the diet of humans and other mammals. The naturally occurring and synthetic derivatives of vitamin A (retinol) are collectively referred to as retinoids. Retinol and its active metabolites and/or derivatives are needed for vision, growth, reproduction, cell proliferation, cell differentiation, and the integrity of the immune system (Dawson 2000). The major established pathway of retinol activation involves mobilization of retinyl esters, reversible conversion of the released retinol into retinal, and irreversible conversion of retinal into retinoic acid (Napoli 1999). Among the active metabolites, 11-cis-retinal is required by the eye for the transduction of light into neural signals necessary for vision, while all-trans or 9-cis retinoic acid exert most of the other biological functions of vitamin A (Sarri 1994;Denke 2002).

Vitamin A deficiency has been known to occur as a result of poor dietary intake, liver diseases, and gastrointestinal malabsorption (Smith and Steinemann 2000). It is still a major public health problem in over 60 countries worldwide, especially in Africa and South-East Asia. Vitamin A deficiency is the leading cause of preventable blindness in children (World Health Organization 1995). Vitamin A deficiency also increases the risk of disease and death from severe infections.

1.2.1 Intestinal absorption of vitamin A

The intestinal absorption of vitamin A is summarized in Figure 1-1. The major form of vitamin A in a diet is retinyl ester, which is found in foods of animal origin. Small amounts of free retinol and retinoic acid can also be found in foods. Some carotenoids, present in fruit and vegetable, have provitamin A activity and hence can be potential sources of this vitamin (Gottesman et al. 2001).

Before the uptake of dietary vitamin A by enterocytes of the small intestine, dietary retinyl esters are hydrolyzed in the small intestinal lumen. The free retinol is then taken up by enterocytes through a saturable, carrier-mediated process by a yet unidentified retinol transporter when retinol is present at physiologic concentrations, and by passive diffusion when retinol is present at pharmacological levels (450-2700 nmol/L) (Hollander and Muralidhara 1977;Hollander 1981;Quick and Ong 1990). It is proposed that several enzymes, such as pancreatic carboxylester lipase, retinyl ester hydrolase (REH) intrinsic to the brush border membrane of intestine, and pancreatic triglyceride lipase, are responsible for the hydrolysis of retinyl esters in the intestinal lumen.

Pancreatic carboxylester lipase (CEL) (also known as cholesteryl ester hydrolase and bile salt-dependent REH) is secreted by pancreas into the intestinal lumen and its activity is stimulated by trihydroxy bile salt (Harrison 1988;Harrison and Hussain 2001). For decades, this enzyme was thought to be the major enzyme hydrolyzing dietary retinyl esters and other lipids including cholesteryl esters, triglycerides and lysophospholipids *in vivo* (Lombardo and Guy 1980;Blaner and Olson 1994). More recent study, however, suggests that CEL may not be involved in dietary retinyl ester hydrolysis in the small intestine as proposed (Weng et al. 1999). Using CEL knockout mice, results from Weng's study showed that CEL knockout mice and wild-type mice absorbed similar amounts of retinol provided as retinyl ester, while nonhydrolyzable retinyl hexadecyl ether was not absorbed in either knockout mice or wild-type animals. These data confirmed that the hydrolysis of dietary retinyl ester in the lumen of the intestine was necessary prior to its absorption. However, enzyme(s) other than CEL might be responsible for the hydrolysis of dietary retinyl esters in the gut of the mouse.

A bile salt-stimulated REH activity intrinsically located in the brush border membrane of enterocytes of small intestine and stimulated primarily by dihydroxy bile salt has been shown to exist in both rats and humans (Rigtrup and Ong 1992;Rigtrup et al. 1994b). In rats that underwent common-duct ligation 2 days prior to tissue collection in order to prohibit contamination of brush border membrane with enzymes secreted by pancreas, the brush border membrane showed little ability to hydrolyze short-chain retinyl ester (such as retinyl caproate) but retained 70% of long-chain retinyl ester (as retinyl palmitate) hydrolytic activity compared to sham-operated controls (Rigtrup and Ong 1992). The results suggested that short-chain REH was mainly from pancreatic origin, whereas the long-chain REH was mainly intrinsic to the brush border. This intrinsic brush border membrane REH activity in the small intestine of rats was later purified, which showed a potent phospholipase activity and reacted with a polyclonal antiserum against rat brush border phospholipase B (Rigtrup et al. 1994a), suggesting that the intrinsic brush border membrane REH activity is probably due to an intestinal phospholipase B.

Pancreatic triglyceride lipase (PTL) in the intestinal lumen has been found to have bile salt-dependent REH activity (van Bennekum et al. 2000). REH activity was assessed under either CEL-optimal conditions (using millimolar concentrations of trihydroxy bile salt) or PTL-optimal conditions (using millimolar concentrations of dihydroxy bile salt in the presence of saturated concentrations of colipase). Enzyme assays of PTL showed that a colipase-stimulated REH activity existed in the pancreas of CEL-knockout mice, consistent with the hydrolysis of retinyl ester by PTL. These results suggest that PTL is responsible for at least part of the REH activity observed in pancreas homogenates. However, the physiological role of PTL in retinyl ester hydrolysis is yet unclear. Results from that study also showed that pancreatic REH activity was detected not only in the presence of bile salts, but also in the presence of CHAPS, a bile salt analog, and in the absence of bile salts (van Bennekum et al. 2000). Hence, the complete hydrolysis of retinyl esters in the intestinal lumen probably involves several enzymatic activities. The relative roles of each enzyme in the digestion of retinyl esters, however, remain to be determined.

Unlike the dietary retinyl esters, dietary provitamin A carotenoids such as β-carotene are first absorbed into enterocytes by passive diffusion (Hollander and Ruble 1978), then principally converted to retinol via two reactions: 1). Carotenoids are cleaved to retinal by 15,15'-dioxygenase; 2). Retinal is reduced to form retinol by retinal reductase (Blomhoff et al. 1991). The newly absorbed retinol in intestinal enterocytes derived from dietary retinyl esters or carotenoids subsequently binds to the cellular retinol-binding protein II (CRBPII) and is then re-esterified with long-chain fatty acid (mostly palmitate) by lecithin:retinol acyl-transferase (LRAT) (Blaner and Olson 1994).

CRBPII is a 16-kDa polypeptide and is expressed mainly in mature enterocytes in the villi of the small intestine (Crow and Ong 1985). This protein is present in high amounts in the small intestine of rat, representing more than 1% of the soluble protein recovered from the jejunal mucosa (Ong 1985). The binding of retinol to CRBPII is essential for the esterification of retinol by enzyme LRAT since LRAT requires retinol bound to CRBPII as a substrate for the reaction (Quick and Ong 1990;Harrison and Hussain 2001). Another enzyme acyl-CoA:retinol acyltransferase (ARAT) may also play some role in retinol esterification when large amounts of retinol are digested (Blaner and Olson 1994). Unlike LRAT, ARAT re-esterifies only free retinol, but not CRBPII-bound retinol, during the esterification reaction (Ong et al. 1987).

The retinyl esters formed in intestinal enterocytes are then packaged together with triglycerides, free and esterified cholesterol, phospholipids, apolipoproteins, and other fat-soluble vitamins into chylomicrons, transported via lymphatic system into the general circulation (Blomhoff et al. 1991). A small amount of free retinol is also secreted into portal circulation, which may become an essential back-up mechanism for the homoeostasis of vitamin A under certain circumstances (Harrison and Hussain 2001).

In the circulation, the vast majority of chylomicron triglycerides are hydrolyzed by the endothelial cell-bound lipoprotein lipase in extra-hepatic tissues (especially in adipose tissue and skeletal muscle). This process results in the formation of chylomicron remnants, the catabolic end product of chylomicrons (Mahley and Hussain 1991). While chylomicrons undergo lipolysis, retinyl esters are relatively nontransferable and remain associated with chylomicron remnants before they are rapidly removed by the liver (Blomhoff et al. 1991). A small fraction of chylomicron retinyl esters, however, may be transferred to other plasma lipoproteins during chylomicron catabolism (Berr and Kern 1984;Wilson et al. 1983), or cleared by extrahepatic tissues, such as muscle, adipose tissue, kidney, or bone marrow (Blomhoff et al. 1982;Hussain et al. 1989).

1.2.2 Hepatic metabolism of vitamin A

1.2.2.1 Uptake, storage, and mobilization of vitamin A in the liver

The overview of hepatic vitamin A metabolism is shown in Figure 1-2. Despite the large fluctuations in the daily vitamin A intake, the homeostasis of circulating retinol is tightly regulated and maintained within a narrow range, mainly through storing retinyl esters when dietary intake increases, and mobilizing vitamin A from storage when intake decreases in the liver (Blaner and Olson 1994). Two types of hepatic cell - hepatocytes (or parenchymal cells) and stellate cells (or fat-storing cells, lipocytes, and Ito cells) are essentially involved in retinol metabolism in the liver. Hepatocytes account for 60-65% of all cells number and 90% of all protein present in the liver (Blaner and Olson 1994;Blomhoff et al. 1991). These cells are directly involved in the uptake of chylomicron remnants and the synthesis and secretion of RBP (Blaner and Olson 1994). Hepatic stellate cells account for about 6-8% of liver cells and represent less than 1% of liver protein mass (Blomhoff and Berg 1990;Hendriks et al. 1987). Hepatic stellate cells are the major storage cells for retinyl ester in the liver (Blaner and Olson 1994).

As noted earlier, most of the retinyl esters associated with chylomicron remnants in the circulation are rapidly taken up by the liver. Up to 70-80% of labelled chylomicron retinyl esters are recovered in rat liver within 30 minutes after injection (Blomhoff et al. 1982). Hepatocytes in the liver rapidly and efficiently take up components of chylomicron remnants. Several specific hepatic membrane receptors including low density lipoprotein (LDL) receptor, LDL receptor-related protein, lipolysis-stimulated receptor have been proposed to be involved in the hepatic uptake of chylomicron remnants (Cooper 1997).

Following uptake by hepatocytes, the retinyl ester component of chylomicron remnants is hydrolyzed to retinol by REH enzyme. A major fraction of the resulting retinol can be rapidly transferred to stellate cells and stored in the lipid droplets of stellate cells primarily in the form of retinyl esters (Gottesman et al. 2001). This transfer of retinol to stellate cells is apparently influenced by vitamin A status (Blomhoff et al. 1982). When dietary vitamin A is plentiful, the stellate cells account for 70-90% of the vitamin A stored in the liver (Hendriks et al. 1985;Lakshman et al. 1988). The main storage form of retinyl ester is retinyl palmitate (76% to 80%), with smaller amounts of stearate (9% to 12%), oleate (5% to 7%), and linoleate (3% to 4%) (Batres and Olson 1987).

The process by which retinol is transferred between hepatocytes and stellate cells is not completely understood. It is proposed that free retinol in hepatocytes is transported to endoplasmic reticulum via cellular retinol binding protein (CRBP), then bound to newly synthesized RBP in the endoplasmic reticulum, and subsequently secreted from the hepatocytes. Much of the secreted retinol can be taken-up by stellate cells while the remainder presumably escapes the uptake by stellate cells and reaches the blood (Gottesman et al. 2001).

The retinol taken-up by stellate cells needs to be re-esterified to form retinol esters to be stored in the cells. In both hepatocytes and stellate cells, LRAT, ARAT enzyme activities and CRBP are detected (Blomhoff et al. 1985;Blaner et al. 1985a). Similar to the CRBPII in the intestine, CRBP specifically binds intracellular retinol with great affinity. CRBP-bound retinol is the preferential substrate for LRAT in the liver. Under normal physiological condition, retinol in hepatic cells binds with CRBP, which directs retinol to LRAT for esterification, whereas ARAT esterifies the excess retinol in its free form when retinol is present at high levels (Yost et al. 1988). Once retinol is needed by extra-hepatic tissues, retinyl esters stored in the stellate cells are hydrolyzed by enzyme REH to release retinol.

1.2.2.2 Hepatic retinyl ester hydrolysis

In the process of hepatic vitamin A metabolism, hydrolysis of retinyl esters plays an important role. Both the chylomicron remnant uptake by hepotocytes and the mobilization of stored retinyl esters from the liver require retinyl ester hydrolysis. Although numerous studies have been focused on the enzymatic hydrolysis of retinyl esters, much remains to be known about the enzymology of this reaction. The physiological roles of the enzymatic activities involved in the hydrolysis of retinyl esters *in vivo* are still not clear. So far, several biochemically distinct retinyl ester hydrolase (REH) activities have been described in various subcellular fractions of the liver (Harrison 1998). These include bile salt-dependent REH, CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase.

- *Bile salt-dependent REH and CHAPS-stimulated REH*

Bile salt-dependent REH has been extensively investigated in the past decades. This enzymatic activity requires millimolar concentrations of bile salts for maximal *in vitro* activity (Prystowsky et al. 1981;Harrison 1988). The enzyme has a neutral pH optimum and is capable of catalyzing the hydrolysis of a variety of substrates, including cholesteryl ester, triglyceride and retinyl ester (Harrison 1998). Among the substrates, bile salt-dependent REH has higher affinity and specific activity for cholesteryl esters and triglycerides than for retinyl ester (Blaner et al. 1984;Prystowsky et al. 1981). One study showed that retinyl ester was hydrolyzed at about one-fifth the rate of cholesteryl oleate under similar assay conditions (Harrison and Gad 1989). Interestingly, this enzyme activity has an unusual property of being highly variable among individual animals. The variability of bile salt-dependent REH activity has been observed to vary more than 50-fold in individual rat liver and the activity variation among the individuals is not related to the age or vitamin A status of the animals (Prystowsky et al. 1981). This high variability could be the result of sampling since results from another study found the enzyme had a significant section-to-section variation, which did not exhibit any consistent anatomical pattern from animal to animal (Blaner et al. 1985b)

The bile salt-dependent REH enzyme has been reported to be present broadly in both hepatocytes and stellate cells in the liver (Blaner et al. 1985a;Blomhoff et al. 1985). The cellular distribution of this enzyme is uniform throughout normal rat liver on the basis of cell protein (Blaner et al. 1985a). However, among subcellular fractions of liver homogenate, the bile salt-dependent REH activity is unusually distributed, equally between the low-speed nuclear fraction and the high-speed supernatant, with very low activity being encounted in microsomes (Harrison and Gad 1989).

It has been suggested that hepatic bile salt-dependent REH has a strong similarity to the CEL (also designated bile salt-activated lipase, or cholesteryl ester hydrolase) of pancreatic origin. The properties of the REH activity from the liver are nearly identical to those of purified rat pancreatic CEL such as the dependence on pH, cholate, and substrate concentration (Harrison 1988;Harrison and Gad 1989). The similarity of both enzymes is further suggested by the fact that antibodies to the pancreatic enzyme specifically inhibit the bile salt-dependent REH activity and CEL activity in the liver of rat (Harrison 1988). In addition, by molecular cloning of a rat complementary deoxyribonucleic acid (cDNA) encoding the hepatic enzyme, it has been revealed that the cDNA for liver bile salt-dependent retinyl ester/cholesteryl ester hydrolase has the same sequence as mature pancreatic CEL (Chen et al. 1997).

So far, little is know about the physiological role that bile salt-dependent REH plays in the hepatic uptake of chylomicron remnant-associated retinyl ester and/or mobilization of retinyl ester from the liver. In one study, it has been reported that CEL had no effect on hepatic uptake of chylomicron remnant-derived retinyl ester *in vivo* (van Bennekum et al. 1999). This study compared the serum clearance and hepatic uptake of chylomicron remnant-derived retinyl ester between CEL gene knockout mice and wild-type mice. All the mice were killed 30 min after the intravenous injection of [^{3}H]-retinyl ester from chylomicrons. Results showed that serum clearance rates of radioactivity label were not different between wild-type and CEL-deficient mice. No significant change in radio labeled-retinol appearance was observed in the liver of CEL deficiency and wild-type animals 30 min after the injection. In addition, total vitamin A and free retinol levels in the liver and some other tissues, as well as the retinol level and RBP in serum, were not different between wild-type and CEL-deficient mice. These data indicate that the lack of CEL expression affects neither the uptake of dietary chylomicron associated retinyl ester by the liver, nor the hepatic vitamin A metabolism. Interestingly, the hepatic bile salt-dependent REH activity is similar in both wild-type and CEL-deficient mice. All these results point to the fact that another bile salt-dependent REH enzyme activity, distinct from CEL, may be present in the liver of the mice.

Using 50-300 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 0.2% Triton X-100 as detergent rather than bile salt in the assay mixture, a CHAPS-stimulated REH activity has been described in the livers of pigs (Cooper and Olson 1986;Cooper and Olson 1988), rats (Cooper et al. 1987;Tsin et al. 1986), and humans (Mourey and Amedee-Manesme 1992). CHAPS is a bile salt analog, has zwitterionic character opposed to the anionic character of sodium cholate, and superior ability to solubilize and stabilize protein (Cooper et al. 1987). The optimal pH range of CHAPS-stimulated REH is about 7-9 (Schindler et al. 1998). Compared with the radiometric assay of bile salt-dependent REH in other studies (Prystowsky et al. 1981;Harrison 1988), much higher concentrations of substrate and enzyme protein are used to measure CHAPS-stimulated REH activity (Cooper et al. 1987).

The high inter-animal variability reported in bile salt-dependent REH activity is relatively low in CHAPS-stimulated REH. The CHAPS-stimulated REH is reported a 3-fold variability in pig liver homogenates and a 5-fold in rat liver homogenates (Cooper and Olson 1986;Cooper et al. 1987). This lower inter-animal variability observed in CHAPS-stimulated REH may be due to the type and concentration of detergent used in the assay. When CHAPS was replaced with equimolar sodium cholate, or with reduced concentrations of CHAPS and substrate retinyl palmitate, the REH activity was more variable (Cooper et al. 1987). CHAPS may actually stabilize REH, and the high substrate concentration in the assay system may provide a more consistent hydrolytic rate than when lower concentrations near the apparent *K*m are present (Cooper and Olson 1986).

Studies also showed that about one-third of CHAPS-stimulated REH activity in both pigs and rats was found in the absolute supernatant fraction of liver homogenates, with the remainder being in the membrane fractions (Cooper and Olson 1986;Cooper et al. 1987). There is no evidence indicating that the bile salt-dependent REH, as mentioned earlier in this review, is physiologically related to CHAPS-stimulated REH except that the activities of both enzymes could be inhibited by α-tocopherol in the reaction mixture (Prystowsky et al. 1981;Napoli et al.

1984;Schindler et al. 1998). Sequence analysis revealed that the CHAPS-stimulated REH protein is distinct from bile salt-activated lipase and cholesterol esterases (Schindler et al. 1998). More work is warranted before one can fully understand the physiological roles and characteristics of bile salt-dependent REH and CHAPS-stimulated REH *in vivo*.

- *Bile salt-independent REH*

Retinyl ester hydrolysis is observed in the absence of cholate in homogenates of rat liver, lung, kidney, intestine, and testes (Napoli et al. 1989). Studies using subcellular fractions prepared by differential centrifugation of rats liver homogenates demonstrate that the microsomal fraction has significantly higher enzymatic activities of both neutral and acid bile salt-independent REH (optimal pH 7 and 5, respectively) (Gad and Harrison 1991;Harrison et al. 1995). This microsomal fraction is highly enriched in plasma membranes/endosomes as assessed by the enrichment of alkaline phosphodiesterase, an enzyme marker for such organelles (Gad and Harrison 1991).

These neutral and acid bile salt-independent REH activities are also distincit from the previously described bile salt-dependent REH in several other aspects. The enzymatic activities of bile salt-independent REH neither vary markedly among individual rats nor are they inhibited by antibodies to pancreatic CEL as shown in bile salt-dependent REH (Harrison and Gad 1989;Gad and Harrison 1991). Additionally, neutral bile salt-independent REH shows specificity for retinyl esters over triglycerides and this enzyme does not catalyze the hydrolysis of cholesteryl esters (Napoli et al. 1989;Sun et al. 1997). The specificity of bile salt-independent REH suggests that the enzyme may be important in hepatic retinyl ester metabolism (Sun et al. 1997).

Several studies were conducted to explore the possible roles of bile salt-independent REH in the hepatic metabolism of vitamin A. Results from one of these studies showed that labelled retinyl esters were rapidly cleared from plasma and appeared in the liver after the intravenous injection of chylomicrons containing tritium-labelled retinyl esters into rats (Harrison et al. 1995). Within the liver, label first appeared in the plasma membrane/endosomal fraction, which is enriched in neutral and acid bile salt-independent REH activities. The co-localization of newly delivered chylomicron retinyl esters and bile salt-independent REH activities in the same plasma membrane/endosome fraction, and the *in vitro* hydrolysis of chylomicron remnant retinyl esters by these fractions, suggest that both the acid and neutral REH function in the initial hepatic uptake of chylomicron remnant associated retinyl esters.

The cellular distributions and relative specific activity values (defined as the percentage of recovered activity divided by the percentage of recovered protein) of the neutral and acid bile salt-independent REH does not reveal any preferential enrichment in either the hepatocyte or nonparenchymal cell fractions (Matsuura et al. 1997). The acid REH is shown to hydrolyze retinyl esters present in the lipid droplets of hepatic stellate cells in rats *in vitro* (Azaïs-Braesco et al. 1995). The cell localization of both neutral and acid bile salt-independent REH suggests that these enzymes might have an important role in mobilizing stored vitamin A esters in hepatic stellate cells.

Bile salt-independent REH activity does not differ between vitamin A-sufficient and –deficient rats (Matsuura et al. 1997). The retinyl ester hydrolysis catalyzed by bile salt-independent REH is stimulated by apo-CRBP, which binds the freed retinol and facilitates its transport within the liver, suggesting that apo-CRBP is a signal for retinyl ester mobilization (Boerman and Napoli 1991). Unlike bile salt-dependent REH and CHAPS-stimulated REH activity that are inhibited by α-tocopherol *in vitro* (Napoli et al. 1984;Schindler et al. 1998;Prystowsky et al. 1981), bile salt-independent REH activity does not respond to various α-tocopherol concentrations (10, 100 or 500 μM) in the assay system (Boerman and Napoli 1991). Additionally, the presence of zinc acetate or zinc chloride (1mM) can result in a greater than 80% inhibition of the neutral bile salt-independent REH activity without affecting the acid bile salt-independent REH activity *in vitro* (Gad and Harrison 1991;Boerman and Napoli 1991).

- *Non-specific carboxylesterases*

As a group, non-specific carboxylesterases hydrolyze a wide range of ester substrates including cholesteryl esters, retinyl esters, acyl coA, and monoacyl glycerols (Mentlein et al. 1987;Mentlein et al. 1984). Six carboxylesterases have been purified from rat liver microsomes and are referred to (according mainly to their isoelectric points) as alloenzymic forms of ES-2 (serum esterase), ES-3 (pI 5.6 esterase), ES-4 (pI 6.2/6.4 esterase or microsomal hydrolase), ES-10 (pI 6.0/6.1 esterase), and ES-15 (pI 5.0/5.2 esterase) (Mentlein et al. 1987). These isozymes share several molecular properties, such as similar subunit weights, low carbohydrate content, one active site per protein subunit, presence of serine in the active site and selective sensitivity to inhibition by bis-4-nitrophenyl phosphate. All of these enzymes have polypeptide monomer molecular masses of 58-65 kDa and function catalytically as the monomer, except for esterase ES-10, which exists as a homotrimer in the native state (Mentlein et al. 1987).

The activities of non-specific carboxylesterases are mainly localized in hepatocytes in the liver although activities are also present in non-parenchymal cells (Gad 1994). Non-specific carboxylesterases are remarkably affected by dietary vitamin A status. A vitamin A deficient diet results in significant decreases in carboxylesterase activities in hepatocytes but does not affect the enzyme activity in non-parenchymal cells in the liver (Gad 1994).

Sequence analysis reveals that non-specific carboxylesterases are similar to CHAPS-stimulated REH protein (Schindler et al. 1998). Among the isozymes of non-specific carboxylesterase, ES-4 is the one that hydrolyzes retinyl ester in the liver (Mentlein and Heymann 1987). The activity of ES-4 is enriched in endoplasmic reticulum of rat liver cells (Mentlein and Heymann 1987). Such cell localization of ES-4 suggests that ES-4 may be involved in the mobilization of retinol from the vitamin A esters stored in the liver. The structural, immunological and catalytic features, pH dependence, and the inhibition effect of bis-(4-nitrophenyl) phosphate on ES-4 are similar to those reported for CHAPS-stimulated REH in pig (Schindler et al. 1998). However, the non-specific carboxylesterase ES-4 differes from CHAPS-stimulated REH in molecular mass (61 kDa and 70kDa, respectively) and the requirement of CHAPS in the assay system (Schindler et al. 1998). Analysis of the amino acid sequences shows that a purified bile salt-independent REH from hepatic microsomes is highly homologous with other hepatic carboxylesterases, in particular, ES-2 in the liver (Sun et al. 1997). Antibodies against ES-2 react with the purified neutral REH. In addition, ES-10 may function as a bile salt-independent REH in rat liver (Sun et al. 1997). It is still not clear, however, if ES-2 or ES-10 are identical to bile salt-independent REH, or they are distinct enzymes involved in the metabolism of vitamin A in the liver.

1.2.2.3 Hepatic retinol esterification

Hepatic retinol needs to be esterified with long-chain fatty acids (predominantly palmitic, stearic and oleic acids) before it is stored in the liver in the form of its esters. Retinol esterification is thus a key reaction that regulates retinol intracellular availability. Two retinol-esterifying enzymes, LRAT and ARAT, have been identified in the microsomal fraction of the liver, and some other tissues including small intestine, retinal pigment epithelium, lung, and testes. (Randolph et al. 1991;Ross 1982;Sarri and Bredberg 1988;Helgerud et al. 1982;Schmitt and Ong 1993;Zolfaghari and Ross 2002). LRAT transfers fatty acid from lecithin to free retinol or

CRBP-bound retinol complex (MacDonald and Ong 1988;Yost et al. 1988;Randolph and Ross 1991). Although LRAT can use both free and CRBP-bound retinol as substrate, it has higher affinity for CRBP-retinol complex (Randolph et al. 1991;Yost et al. 1988;Ong et al. 1988). In contrast to LRAT, ARAT depends upon acyl-CoA for retinyl ester synthesis (Ross 1982). This enzyme catalyzes the esterification of free retinol only (Randolph et al. 1991). Due to the hydrophobic nature of retinol, hepatic retinol is always bound to CRBP within liver cells. Therefore under normal physiologic conditions, LRAT is always the major enzyme contributing to retinol esterification in the liver (Ruiz et al. 1999). ARAT may participate in retinyl ester production when free retinol concentration is higher than that of CRBP (Randolph and Ross 1991). Moreover, in tissues that do not synthesize CRBP, such as mammary gland, ARAT is the only physiologically active enzyme that esterifies retinol (Randolph et al. 1991).

Both LRAT and ARAT activities are present in hepatocytes and hepatic stellate cells. The higher specific activities are found in the stellate cells (Blomhoff et al. 1991). A cellular distribution study found that the specific activity of LRAT was approximately 10-fold greater in the hepatic nonparenchymal cells than that in hepatocytes in vitamin A-sufficient rats (Matsuura et al. 1997). This cellular localization of enzymes is consistent with the high levels of retinol ester in hepatic stellate cells and indicates that LRAT plays an important role in hepatic vitamin A storage.

Several studies indicate that hepatic LRAT activity is regulated by vitamin A status. In retinol-depleted rats with undetectable liver vitamin A stores and low plasma retinol concentrations, hepatic LRAT activity was almost undectable (Randolph and Ross 1991;Matsuura et al. 1997). Similarly, LRAT mRNA is virtually absent from the liver of vitamin A-deficient rats and mice (Zolfaghari and Ross 2000). The depressed LRAT protein and mRNA levels in vitamin A-deficient animals can be quickly recovered by dietary retinol or all trans-retinoic acid (Randolph and Ross 1991;Zolfaghari and Ross 2000). The regulation of LRAT is highly tissue-specific: neither the LRAT activity or LRAT mRNA in the intestine is regulated by vitamin A status or dietary vitamin A supplements (Randolph and Ross 1991;Zolfaghari and Ross 2000). The ability of retinoic acid to regulate hepatic LRAT mRNA expression and activity suggests a closely autoregulated retinoid homeostasis in which retinoic acid serves to regulate vitamin A storage in liver (Zolfaghari and Ross 2000). Hepatic ARAT activity, on the other hand, does not vary significantly with the vitamin A status of the animal (Randolph and Ross 1991).

1.2.2.4 Cellular retinol binding protein in liver (CRBP)

Cellular retinol binding protein is a 15 kDa protein and belongs to a family of small intracellular proteins known as the intracellular lipid-binding protein family, which also includes CRBPII, cellular retinoic acid-binding protein type I and type II (Noy 2000). The primary sequences of rat CRBP and CRBPII show 56% identity (Noy 2000) although the two proteins display a strikingly different tissue distributions. CRBPII is located primarily in the intestine in adults, while CRBP is highly expressed in the liver, kidney, lung, brain, retinal pigment epithelium and other tissues (Eriksson et al. 1984;Zetterström et al. 1994;De Leeuw et al. 1990). In the liver, CRBP is present in both hepatocytes and stellate cells of vitamin A sufficient rats. However, when the CRBP levels are normalized to cellular protein content, the concentration of CRBP in liver stellate cells is much higher than that in hepatoctyes (Blomhoff et al. 1991).

In hepatic cells, apo-CRBP specifically binds intracellular retinol or retinal with great affinity (Fortuna et al. 2001). One earlier study examined the subcellular distributions of CRBP in the livers of rats fed either vitamin A-containing or vitamin A-deficienct diets to obtain a total vitamin A storage range from 0.5 to 172 μg of retinol equivalent (RE) per gram of liver (Harrison et al. 1987). Results showed that, at all levels of liver retinol content, there was sufficient CRBP to account quantitatively for the amounts of free retinol present in the soluble fraction of the cells (Harrison et al. 1987). It is thus suggested that CRBP might function as a cytosolic transport protein to direct retinol to various cellular sites of metabolism. By capturing retinol from cytosol, CRBP also helps to maintain the low concentrations of potentially membrane-disruptive free retinol inside the cell (Noy and Blaner 1991).

CRBP is involved in the regulation of vitamin A storage in the liver. The CRBP-retinol complex (holo-CRBP) is the substrate for retinyl ester synthesis catalyzed by LRAT (Ong et al. 1988), whereas apo-CRBP strongly inhibits this reaction (Herr and Ong 1992). Moreover, apo-CRBP stimulates the hydrolysis of retinyl esters by REHs (Boerman and Napoli 1991). It is thus hypothesized that CRBP binds and directs retinol to LRAT to be esterified and then stored as retinyl esters if vitamin A is sufficient. However, if vitamin A is deficient, the levels of retinol and holo-CRBP in the liver are decreased, while the apo-CRBP level inside hepatic cells is increased. An increased apo-CRBP level will therefore inhibit the esterification of retinol by LRAT, and accelerate the hydrolysis of stored retinyl esters by REH, thereby allowing retinol to be released into the circulation (Noy 2000). This hypothesis is supported by a study showing that the retinyl ester level in hepatic stellate cells in CRBP gene knockout mice was 50% lower than that in wide-type mice (Ghyselinck et al. 1999). The same study also suggested that the decreased capacity to maintain retinyl ester stores in CRBP-deficient mice might not be due to the alteration of the enzyme activities involved in the synthesizing and hydrolyzing retinyl esters. The hepatic LRAT or ARAT activities, as well as the neutral and acid bile salt-dependent REH activities, were similar between CRBP-deficient mice and wild-type animals.

The CRBP-retinol complex is also involved in the synthesis of retinoic acid. In the process of reitnoic acid synthesis, retinol is first oxidized to form retinal by the enzyme retinol dehydrogenase, which recognizes CRBP-bound retinol in the cells (Boerman and Napoli 1995). The resulting retinal is then bound to apo-CRBP and converted to retinoic acid via the action of retinal dehydrogenase type 1 or type 2 (Napoli 1999). Apo-CRBP modulates retinoic acid metabolism by inhibiting retinol dehydrogenase as well as retinal dehydrogenase type 1 (Napoli 2000).

1.2.3 Retinol binding protein and transthyretin

RBP is a single polypeptide with a molecular weight of about 21 kDa, and possesses one binding site for one molecule of retinol. RBP mRNA and/or RBP protein are synthesized in many tissues including the liver, kidney, adipose tissue, lung, heart, spleen, and eyes (Soprano and Blaner 1994;Noy 2000). The liver is the major site of RBP synthesis and secretion in the body. The only known physiological function of RBP is in the delivery of retinol to various tissues (Vogel et al. 1999).

It has been demonstrated that hepatic synthesis of RBP is not affected by vitamin A status (Blomhoff et al. 1991). However, the secretion of RBP from the liver is tightly regulated by the availability of retinol. Under normal physiological condition, RBP binds to retinol liberated from its hepatic storage pools before the RBP-retinol complex is secreted into the general circulate (Soprano and Blaner 1994). In the state of vitamin A deficiency, RBP secretion is inhibited and therefore this protein accumulates in the hepatocytes (Noy 2000). Thus RBP is directly involved in the mobilization of retinol from the liver.

Absence of RBP in mice dramatically reduces serum retinol levels, but that does not impair the accumulation of hepatic retinol storage in the animals. Research found that RBP-gene knockout mice had significantly lower blood retinol levels (12.5% of the content in wild-type) during the first month of life (Ghyselinck et al. 1999). By contrast, mice from both groups had equivalent hepatic total retinol concentrations at this age. At the age of 5 months, the RBP-deficient mice had accumulated remarkedly higher hepatic retinyl esters concentrations compared to wild-type mice although serum retinol levels in the knockout mice remained low. It was thus suggested that the RBP-deficient mice were unable to mobilize retinol from hepatic storage pools.

The site of retinol mobilization in the liver and the relevant mechanisms have not been clarified. On one hand, hepatocytes are thought to be the only site of retinol mobilization from the liver. That is based on the findings that hepatocytes are able to synthesize and secrete large amounts of RBP, while only trace amount of RBP and no RBP mRNA have been detected in hepatic stellate cells (Soprano and Blaner 1994). A study has reported that hepatic stellate cells express immunoreactivity against RBP when cultured in a medium containing RBP but such cells are unable to synthesize RBP transcripts and proteins (Sauvant et al. 2001). Results from the same study also showed that cellular contacts between hepatocytes and stellate cells greatly enhanced the transfer of retinol from stellate cells. These results suggest that retinol could be transferred from stellate cells back to hepatocytes through cellular contacts between hepatocytes and stellate cells (Sauvant et al. 2001). However, results from other studies showed that the stellate cells isolated from the liver contained RBP mRNA and the cultivated stellate cells were able to secrete RBP to the medium (Andersen et al. 1992;Senoo et al. 1990). Data from Andersen's group (1992) demonstrated that radioactive retinol is mobilized as a retinol-RBP complex in cultivated stellate cells loaded with radio-labeled retinyl ester. These findings suggest that retinol may be mobilized directly from the stellate cells to blood as holo-RBP, whereas the RBP is either the product of endocytosis of plasma RBP, or is synthesized within stellate cells (Andersen et al. 1992).

No matter where and how the liberated retinol binds to its binding protein RBP, once the retinol-RBP complex is formed, it moves to the Golgi apparatus. There the retinol-RBP complex is secreted into the general circulation and circulates with another serum protein, transthyretin (TTR), at a 1:1 molar ratio (Blaner and Olson 1994). TTR is mainly synthesized and secreted by the liver and choroid plexus. It exists as a 55-kDa tetramer of identical subunits in the circulation (Wei et al. 1995). Besides transport of RBP-retinol, TTR also transports thyroid hormone in the body. Binding of RBP-retinol to TTR prevents the loss of the smaller protein -RBP- from the circulation by filtration in the renal glomeruli (Noy 2000). In TTR gene knockout mice the plasma retinol and RBP levels are very low (6% and 5% compared to wild type animals). However, the retinol and retinyl ester levels in the liver and other tissues including kidney, testis, and spleen are similar for mutant and wild type mice (Wei et al. 1995). Further study found that the low circulating levels of retinol-RBP in TTR-deficient mice arose from increased renal filtration of the retinol-RBP complex in these animals (van Bennekum et al. 2001).

1.2.4 Vitamin A in extra-hepatic tissues

The complex of retinol-RBP-TTR transports retinol in the circulation and finally delivers retinol to target tissues where it supports vitamin A-dependent functions. Retinol enters most of its target cells in its free form, unaccompanied by the binding proteins (Soprano and Blaner 1994;Vogel et al. 1999). The mechanism through which tissues and cells take up retinol from the circulating retinol-RBP-TTR complex is not fully established. It has been proposed that a RBP receptor embedded in the plasma membranes of target cells mediates transfer of retinol from RBP across the membrane and into cytosol or directly to CRBP in the cells (Noy 2000). The resultant apo-RBP is then released and subsequently metabolized in the kidney (Blaner and Olson 1994). Alternatively, it is suggested that cellular uptake of retinol from RBP in blood occurs spontaneously and follows the concentration gradients of free retinol (Noy 2000).

Within tissues, retinol is oxidized to form retinoic acid intracellularly. As a major active form of vitamin A, retinoic acid plays important roles by binding to specific nuclear receptors to regulate transcription of target genes including the genes that encode hormones, growth factors, transcription factors, membrane receptors, extracellular matrix proteins, structural proteins, and enzymes involved in diverse metabolic processes (Mangelsdorf et al. 1994). Retinoic acid is hence required for the differentiation, growth, and functions of the cell (Clagett-Dame and Plum 1997).

In the eye, vitamin A has essentially two roles: a) it synthesizes and regenerates the visual pigment rhodopsin in the retina; and b) it is involved in conjunctival epithelial cell RNA and glycoprotein synthesis, which help to maintain the conjunctival mucosa and corneal stroma (Smith and Steinemann 2000). Low vitamin A levels in the eye will lead to a loss of mucous-secreting goblet cells and eventually to squamous cell metaplasia of the conjunctival epithelial cells, which causes the dryish and sandy-like changes of the conjunctiva (Smith and Steinemann 2000;Sommer 1998). Severe vitamin A deficiency leads to corneal ulceration and keratomalacia. The latter is the end stage of the disease representing progressive necrosis of the cornea and invariably results in permanent blindness (Reddy 1991).

1.2.5 Plasma retinol homeostasis

It is estimated that about 95.5% of plasma retinol is present as the retinol-RBP-TTR complex, 4.4% as retinol-RBP, and very little as unbound retinol (Green and Green 1994). The retinol in plasma is not only used by target tissues, but also recycled back to the liver (Blomhoff et al. 1991). Under normal physiological conditions, plasma retinol level is homeostatically regulated and maintained within a narrow concentration range by factors affecting the balance between retinol input to plasma and retinol output from plasma. Input of retinol to plasma depends on retinyl ester hydrolysis, retinol esterification and the ability of retinol to bind to apo-RBP. Output of retinol from plasma involves liver, kidney and other tissues. A number of endogenous and exogenous factors that may alter vitamin A homeostasis have been identified. These include primary vitamin A deficiency, the availability of retinol carrier proteins, protein-energy malnutrition, thyroid status, iron or zinc status, certain liver and kidney disease, inflammation, measles or malarial infection, and other physiological stresses. For example, the levels of both plasma RBP and retinol are reduced to approximately 50% of normal levels in severe protein-energy malnutrition whereas proper feeding can quickly restore these decreased indices in the plasma (Ingenbleek et al. 1975;Smith et al. 1973).

The liver and kidneys are the two major organs playing key roles in plasma retinol homeostasis. As noted earlier, liver is not only the dietary vitamin A processor, but it is also a vitamin A storage site. Additionally, liver is one of the routes for plasma retinol output. It has been shown that both acute and chronic liver disease can lead to decreased plasma levels of retinol and RBP in humans (Green and Green 1994). The kidneys also play an important role in vitamin A metabolism including the glomerular filtration of the plasma retinol-RBP complex and apo-RBP, and the reabsorption of RBP in the convoluted proximal tubules (Kato et al. 1984). It has been reported that plasma retinol levels are increased in patients with certain renal diseases that result in severe decreases in glomerular filtration rate (Blomhoff et al. 1991). In acute infection, a significant amount of retinol is excreted in the urine. That is probably caused by an impaired tubular reabsorption of low-molecular-weight proteins, such as RBP (Mitra et al. 1998).

1.3 Relationships between zinc and vitamin A, type 1 diabetes

Zinc is another important factor involved in the regulation of plasma retinol homeostasis. The role of zinc in vitamin A metabolism is of particular interest because of the potential relationship between zinc and diabetes.

Zinc is essential to the functions of numerous proteins, which includes its role as a component of a catalytic site of hundreds of enzymes. Zinc metalloenzymes have been found in every known class of enzymes – oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (McCall et al. 2000). Zinc is also involved in the deoxyribonucleic acid (DNA) transcription process as well as gene expression through the so-called "zinc finger" motif, which is the most common recurring motif in transcription proteins (Hambidge 2000). These affect cellular growth and differentiation.

The diverse roles of zinc in metabolic processes are reflected in the wide variety of symptoms which appear when cellular zinc supply is inadequate. Mild zinc deficiency leads to impaired growth or growth failure, impaired wound healing, dermatitis, night blindness, appetite changes, diarrhea, pneumonia, etc. More severe zinc deficiency affects organ systems including the epidermal, gastrointestinal, central nervous, immune, skeletal and reproductive systems (Hambidge 2000).

1.3.1 Zinc in vitamin A metabolism

Zinc participates in the absorption, mobilization, transport, and metabolism of vitamin A. A reduction of plasma vitamin A levels in zinc-deficient animals has long been reported (Smith, Jr. 1980). Previous studies found beneficial effects of zinc supplementation on vitamin A metabolism in malnourished children or preterm infants (Shingwekar et al. 1979;Hustead et al. 1988). A study in Mexican preschool children found that, six months after providing zinc supplementation (20 mg/d), the plasma retinol levels were significantly increased in the children compared with the placebo group (Muñoz et al. 2000). This increase was greater in children with vitamin A or zinc deficiency at the beginning of the study (Muñoz et al. 2000).

The underlying mechanisms for the role of zinc in vitamin A metabolism have not been completely clarified. However with rats it has been shown that zinc deficiency may affect the absorption of vitamin A by decreasing lymphatic absorption of retinol (Ahn and Koo 1995). Such reduced vitamin A absorption in zinc-deficient rats is highly correlated with a decrease in lymphatic phospholipid output, which probably links to an impaired biliary secretion of phospholipid into the intestine (Ahn and Koo 1995). Moreover, it has been suggested that zinc deficiency interferes with vitamin A metabolism by decreasing RBP synthesis in the liver and thus leads to a lower concentration of RBP in the plasma (Christian and West 1998). One study showed that hepatic retinol-binding protein synthesis required for mobilization of retinol from its hepatic store was dependent on an adequate level of zinc in rats (Smith et al. 1974). Decreased plasma RBP levels have been found in rats fed a zinc-deficient diet (10 mg zinc/kg diet) compared with rats fed a control diet (25 mg zinc/kg diet) (Kelleher and Lönnerdal 2001). However, no change in hepatic RBP mRNA level has been observed in rats fed a zinc-deficient diet (Kelleher and Lönnerdal 2001). This hypothesis is not supported by some other studies. It was reported that the relative abundance of RBP mRNA was increased in both zinc-deficient and pair-fed rats (Kimball et al. 1995). In cultured HepG2 cells, results further indicated that RBP mRNA and protein concentrations were increased as the media zinc concentration decreased (Satre et al. 2001). The elevated RBP mRNA level was reversed by switching cells from zinc-deficient to zinc-supplemented media (Satre et al. 2001). As a result, the authors suggested that the increase of RBP might serve to facilitate secretion of retinol into the circulation as part of an early compensatory response to a low zinc state (Satre et al. 2001). Furthermore, retinol transport within the hepatic cells is probably impaired in zinc deficiency because of the fact that hepatic CRBP level is reduced by more than 50% in rats fed a zinc-deficient diet compared to the control animals (Mobarhan et al. 1992).

In addition to its roles on RBP- and/or CRBP-mediated intercellular and intracellular transport of retinol, zinc is also involved in the synthesis of enzymes that regulate vitamin A metabolism. Zinc-deficiency significantly decreases the activity of alcohol dehydrogenase and increases the activity of retinal oxidase (Boron et al. 1988). These two enzymes are necessary for the conversion of retinol to retinal, and of retinal to retinoic acid. However, zinc deficiency does not alter the CHAPS-stimulated REH and ARAT activities in the liver (Boron et al. 1988). These changes in hepatic enzyme activities are accompanied by an increase in hepatic vitamin A concentration whereas plasma retinol level remains unchanged. It is therefore suggested that the increased hepatic vitamin A level in zinc deficiency might be partly caused by decreased retinol degradation in the liver.

Moreover, zinc takes part in normal visual functions. It is proposed that decreased alcohol dehydrogenase activity (a zinc metalloenzyme) and depressed protein opsin synthesis in zinc deficiency leads to reduced formation of rhodopsin and eventually causes abnormal dark adaptation (Christian and West 1998). The ocular surface of the conjunctiva and cornea in zinc deficient rat has also been found to be associated with sparse microvilli (Kanazawa et al. 2002). That results in disturbed corneal and conjunctival epithelium and consequently affects visual function.

1.3.2 Zinc in type 1 diabetes

The relationship between zinc and diabetes has long been known. Zinc in the islet cells of pancreas plays a key role in the synthesis, storage and secretion of insulin (Zalewski et al. 1994). In the presence of zinc within the β cell, insulin monomers assemble to a hexameric, crystalline structure for storage and secretion as the zinc crystal (Emdin et al. 1980). When zinc is deficient, there is a decreased insulin content in the pancreatic islet cells of rats (Engelbart and Kief 1970). Zinc has also been shown to have an insulin-like function to stimulate lipogenesis and glucose transport in adipocytes although the mechanism for such function is not yet fully understood (May and Contoreggi 1982). Indeed, zinc-deficient rats show abnormal glucose tolerance and this abnormality can be reversed by repletion of zinc in the animals (Park et al. 1986).

Zinc also has antioxidant functions such as protection against vitamin E depletion, stabilization of membrane structure, restriction of endogenous free radical production, contribution to the structure of the antioxidant enzyme superoxide dismutase (SOD), and maintenance of tissue concentrations of metallothionein (DiSilvestro 2000). It is suggested that zinc may limit oxidant-induced cell damage, a process involved in the pathogenesis of type 1 diabetes and the development of its long-term complications (Chausmer 1998). In diabetic patients, zinc supplementation for 3 to 6 months significantly increases plasma zinc level and decreases oxidative stress, as monitored by thiobarbituric acid reactive substances (TBARS) in the plasma (Roussel et al. 2003).

Zinc supplementation in animals has been shown to delay the onset or the severity of type 1 diabetes. BB (Bio-breeding) rats fed with a diet containing 1000 ppm zinc has a lower incidence of diabetes and improved glucose tolerance at 90 days of age compared with control animals fed a diet containing only 50 ppm zinc (Tobia et al. 1998). High zinc intake also significantly reduces the severity of type 1 diabetes in alloxan and streptozotocin (STZ)-induced diabetic animals based on hyperglycemia, insulin level, and islet morphology (Ho et al. 2001). The authors further suggested that zinc has the ability to up-regulate metallothionein production in pancreatic islets or to modulate the activation of NFκB (a reactive oxygen species-sensitive transcription factor that regulates immune responses) in the diabetogenic pathway, which may be the key mechanism for the protective effect of zinc in diabetes.

Moreover, an increased risk of zinc deficiency has been identified in diabetic patients. Hyperzincuria has been observed frequently in animals and humans with type 1 diabetes (Lau and Failla 1984;Isbir et al. 1994). Studies suggest that hyperglycemia and the resultant glucose load in the kidneys or low insulin level in tissue is responsible for the hyperzincuria in diabetes (Garg et al. 1994;Brandao-Neto et al. 2001). Increased zinc excretion in urine and decreased gastrointestinal absorption of zinc in diabetes might lead to hypozincemia or a decreased tissue store of zinc. However, conflicting results on zinc levels in tissue and plasma have been reported (Chausmer 1998). Some studies reported the same or even higher plasma zinc levels as well as increased tissue zinc in liver, muscle and kidneys in STZ-induced diabetic rats or diabetic BB rats (Brandao-Neto et al. 2000;McNair et al. 1981;Zargar et al. 2002;Cordova 1994) (Failla and Gardell 1985). Change of zinc status may worsen various metabolic abnormalities accompanying diabetes.

1.4 Vitamin A and type 1 diabetes

Type 1 and 2 diabetes are the two major forms of diabetes in humans. Type 1 diabetes is an organ-specific autoimmune disease, resulting from autoimmune destruction of pancreatic β-cells that lead to a total loss of insulin secretion and absolute insulin deficiency in the body. This form of disease typically occurs in childhood, or adolescence, and comprises approximately 5% to 10% of all diagnosed cases of diabetes. Type 2 diabetes has a more complex pathogenesis, which is not yet completely understood. This type of diabetes is characterized by insulin resistance in muscle, liver and adipose tissue and results from relative insulin deficiency in the body. Type 2 diabetes commonly begins after age 40 and accounts for 90% to 95% of all diagnosed cases of diabetes (Centers for Disease Control and Prevention 1997).

Changes of plasma vitamin A level have long been found in diabetic patients (Mosenthal and Loughlin 1944). In type 1 diabetes, vitamin A status has been found to be depressed (Krempf et al. 1991) (Krill et al. 1997) (Granado et al. 1998), whereas in type 2 diabetes, plasma vitamin A concentrations are found to be similar (Straub et al. 1993;Basualdo et al. 1997;Abahusain et al. 1999) or even relatively higher (Havivi et al. 1991;Krempf et al. 1991;Sasaki et al. 1995) compared to healthy controls. It is possible that this difference in vitamin A status between type 1 and type 2 diabetes is partly the reflection of some fundamental differences between these two diseases, such as the insulin level. Indeed, retinol or retinoic acid is required for insulin release in the body. A study has shown that vitamin A-deficient rats have impaired glucose-induced acute insulin release, which can be improved by retinol or retinoic acid repletion (Chertow et al. 1987). Retinol also increases insulin release in β cells of a rat insulinoma cell line, possibly mediated by increased transglutaminase activity in islets (Chertow et al. 1989;Driscoll et al. 1997).

1.4.1 Blindness in vitamin A deficiency and diabetes

Although the importance of altered vitamin A status in diabetes and further linkage between vitamin A and diabetes are not clear, it is known that both vitamin A deficiency and diabetes will lead to a common clinical consequence: impaired visual function or blindness.

The whole spectrum of ocular disease arising from vitamin A deficiency is known as xerophthalmia, which includes night blindness, conjunctival xerosis and Bitot's spots, corneal xerophthalmia, and retinal changes (Sommer 1998). Impaired dark adaptation (or night blindness) is the earliest and most common symptom of vitamin A deficiency. It occurs when retinol is low in photoreceptor rod cells. In the outer segment of rod cells is the visual pigment rhodopsin. The role of rhodopsin in vision function and the regeneration of rhodopsin (so called visual cycle) have been well established (Sarri 1994;Sarri 2000;Baylor and Burns 1998). 11-*cis*-retinal is the light-absorbing chromophore for visual pigments. As a photon of light hits a molecule of rhodopsin, the 11-*cis*-retinal chromophore undergoes isomerization to all-*trans*-retinal. This isomerization leads to a conformational transition within the opsin protein and generates the active photoproduct, metarhodopsin II, which in turn activates a G-protein cascade that generates an electrical response at the surface membrane of rod cells. This response ultimately elicits visual sensations. Before a bleached opsin absorbs another photon, its all-*trans*-retinal must be removed and replaced by 11-*cis*-retinal through the visual cycle. Briefly, the Schiff base linking all-*trans*-retinal and opsin is first hydrolyzed to release free all-*trans*-retinal, which is then reduced to all-trans-retinol. This leaves the rod cells, traverses the interphotoreceptor matrix space bound with interphotoreceptor retinoid-binding protein and enters the retinal pigment epithelium (RPE). All-trans-retinol in RPE is esterified to form all-trans-retinyl ester by the enzyme LRAT and then converted to 11-cis-retinol by an isomerohydrolase. 11-cis retinol can be further esterified by LRAT for storage or oxidized to 11-cis-retinal, which is released from RPE and is taken up by rod cells to be associated with opsin and regenerate the visual pigment (Sarri 1994;Sarri 2000;Baylor and Burns 1998). The visual cycle serves as the only source of rod opsin regeneration (Arshavsky 2002).

In the process of the visual cycle, some of the retinal is lost in this process of visual cycle and a constant source of vitamin A must be available for adequate levels of rhodopsin and optimal rod function to be maintained. Retinol in the circulation enters the visual cycle after it is taken-up from the blood and is esterified to form retinol ester in the RPE. When the circulating levels of retinol are low, the rod function is disrupted and night blindness may occur. Night blindness usually responds rapidly (within days) to systemic administration of vitamin A (Smith and Steinemann 2000).

The pathogenesis of diabetic retinopathy starts with hyperglycemia, which leads to increased levels of protein kinase C, nonenzymatic glycosylation, aldose reductase activity, vasoactive substances, growth factors, and free radicals (Bloomgarden 1999;Cai and Boulton 2002). The earliest clinical manifestations of diabetic retinopathy are classified as nonproliferative diabetic retinopathy. That is characterized by microvascular abnormalites including microaneurysms, intraretinal hemorrhages, and increased retinal vascular permeability. As the disease progresses, gradual loss of the retinal microvasculature occurs and results in retinal ischemia. The more severe form of retinopathy is proliferative diabetic retinopathy manifested by neovascularization, fibrous proliferation, and preretinal and vitreous hemorrhages. With time, the neovascularization tends to undergo contraction, and eventually leads to retinal traction, retinal tear, detachment, and severe visual loss (Neely et al. 1998;Aiello et al. 1998;Ferris et al. 1999). So far, preventing diabetic retinopathy from developing or progressing is the most effective approach to preserve vision in diabetic patients (The Diabetes Control and Complications Trial Research Group 1993).

Although the mechanisms of blindness caused by vitamin A deficiency and diabetes are totally different, it is possible that the altered vitamin A status in diabetic patients can further intensify the retinopathy in this population.

1.4.2 Vitamin A status in type 1 diabetes

In recent years, various studies have focused on the altered vitamin A metabolism in type 1 diabetes. Although there have been sporadic observations showing that plasma retinol levels do not change in type 1 diabetic patients (Hozumi et al. 1998), most of the relevant studies have consistently found a reduction of plasma vitamin A level in the presence of type 1 diabetes. One study has reported significantly lower levels of plasma vitamin A in late adolescent and young adult type 1 diabetic patients compared to age-matched healthy controls (Basu et al. 1989). That result is supported by other studies conducted either in adults (Krempf et al. 1991;Krill et al. 1997;Granado et al. 1998;Merzouk et al. 2003) or children (Baena et al. 2002). Research by Martinoli et al (1993) further demonstrated that decreased plasma retinol levels were found in relatively young type 1 diabetic patients (age < 45 y) with shorter diabetic duration (average 7 y) when compared with age-matched healthy controls. However, the plasma retinol levels did not change in 'older' type 1 diabetic patients (age > 45y) with longer disease duration (average 16 y).

The reduced plasma retinol levels in type 1 diabetic patients reported in the above studies are well within the normal ranges and do not indicate clinical vitamin A deficiency in those patients.

The alteration of plasma vitamin A in type 1 diabetes has been confirmed in diabetic animal models. Rats with STZ-induced diabetes have lower levels of retinol in the plasma and 11-*cis* retinal (a component of rhodopsin) in the eye, despite the fact that those diabetic rats consumed 48% more food compared to that in control counterparts (Tuitoek et al. 1996c). This is also the case in spontaneously developed diabetic BB rats, which have decreased plasma retinol levels in the presence of diabetes compared to non-diabetic control animals (Lu et al. 2000).

1.4.3 Possible cause(s) of disturbed vitamin A status in type 1 diabetes

Reduced plasma retinol levels may be related to a decreased dietary intake of vitamin A, reduced intestinal or hepatic uptake, or depressed mobilization of vitamin A from the liver caused by the alteration of retinol carrier proteins and relevant enzymes. Some other factors such as zinc, iron, insulin level or disease state may also have such effects. Moreover, reduced conversion of carotene to retinol in the body has also been suggested to play a role in the reduced vitamin A level in type 1 diabetes although the result is still inconclusive (Sobel et al. 1953). On the other hand, increased oxidative damage, as well as deficits in antioxidant defense enzymes and vitamins, has been found in the presence of type 1 diabetes (West 2000). Oxidative stress has a potential role in diabetogenesis and the development of diabetic complications. It is thus suggested that the reduced plasma vitamin A concentration might be a consequence of increased consumption of vitamin A resulting from the process of eliminating free redicals in diabetic state (Krill et al. 1997). However, the role of retinol per se as an antioxidant to eliminate free radicals has not been well established.

Contrary to the aforementioned proposals, dietary intake of vitamin A in diabetic patients has been found to be similar to that of the healthy controls (Martinoli et al. 1993;Krill et al. 1997;Basualdo et al. 1997). A pair-fed study of STZ-induced diabetic rats found that the plasma retinol levels were significantly lower in diabetic animals (Tuitoek et al. 1996c). However, the hepatic concentration of total vitamin A remained at the same level as that in the control animals (Tuitoek et al. 1996c). No change in food intake was found between the diabetic BB rats and non-diabetic BB control animals, despite the fact that the diabetic BB rats had significantly lower plasma retinol levels at the onset of diabetes (Lu et al. 2000). These results clearly suggest that the low plasma retinol level in type 1 diabetes is not likely related to the decreased vitamin A intake from the diet.

The uptake of vitamin A in the intestine has been explored by measuring the amount of radio-labeled-retinol taken-up into the jejunum and ileum of STZ-induced diabetic rats *in vitro* (Tuitoek et al. 1994). No alteration in intestinal uptake of retinol was observed in diabetic rats. It was thus concluded by the authors that the intestinal uptake of retinol is not altered by STZ-induced diabetes in rats. It needs to be noted, however, that vitamin A is available mainly as retinyl esters instead of retinol in the diet and these dietary retinyl esters must undergo hydrolysis in the small intestinal lumen before the liberated retinol can be taken up by enterocytes of small intestine. Several enzymes, including pancreatic CEL, REH intrinsic to the brush border membrane of the intestine, and PTL, have been suggested to be involved in the hydrolysis of dietary retinyl ester in the intestine. When dietary vitamin A is sufficient, the availability of liberated retinol prior to intestinal uptake largely depends on the activities of these enzymes. It is not clear, however, if the intestinal uptake of retinyl ester is altered in the presence of diabetes.

As mentioned earlier, the liver is the most important organ involved in the regulation of vitamin A homeostasis. It has been shown that diabetic rats have comparable levels of hepatic total vitamin A concentration whereas the hepatic as well as plasma retinol levels are decreased in the presence of diabetes (Lu et al. 2000). Those results suggest that decreased plasma retinol levesl in diabetes are more likely related to hepatic retinol mobilization, but not to the hepatic uptake and storage of this vitamin. Hepatic mobilization of vitamin A includes the hydrolysis of retinyl ester from its hepatic store to liberate retinol, the binding of freed retinol to RBP to form holo-RBP, and the release of holo-RBP to the circulation, whereby retinol is transported to various tissues as retinol-RBP-TTR complex. In parallel with the reduced level of plasma retinol in type 1 diabetes, a decreased level of RBP and TTR has been reported in both diabetic patients and diabetic rats (Basu et al. 1989;Krill et al. 1997;Tuitoek et al. 1996b;Lu et al. 2000). Hepatic RBP concentration and its mRNA level are also reduced in diabetic BB rats at the onset of diabetes (Lu et al. 2000). Thus it seems that decreased synthesis and/or secretion of RBP in the presence of diabetes might result in a reduced vitamin A mobilization from the liver and contribute partly to the altered plasma vitamin A level in the diabetic state.

The decreased synthesis and/or secretion of RBP in type 1 diabetes might be related to the disturbed zinc status in the disease state. Decreased serum zinc levels, likely the result of hyperzincuria, have been reported in type 1 diabetes (Garg et al. 1994;Isbir et al. 1994). As stated earlier, zinc deficiency might interfere with vitamin A metabolism by decreasing hepatic synthesis of retinol carrier proteins, such as RBP and CRBP (Christian and West 1998;Mobarhan et al. 1992). Zinc deficiency also leads to the alteration of some enzyme activities such as alcohol dehydrogenase and retinal oxidase in vitamin A metabolism (Boron et al. 1988). In diabetic BB rats, reduced retinol levels, RBP concentration sand decreased zinc levels in plasma co-exist at the onset of diabetes (Lu et al. 2000). It is therefore suggested that disturbed zinc status in type 1 diabetes may contribute to the impaired vitamin A metabolic availability by reducing the synthesis or secretion of retinol carrier proteins in the liver.

Attempts have been made to explore the possible restoration of decreased vitamin A level and/or RBP concentration in type 1 diabetes by dietary intervention. However the decreased plasma vitamin A levels do not appear to respond to dietary zinc or vitamin A supplementation in the rats. Dietary zinc (120 μg/g diet) or vitamin A (36 retinol equivalents /g diet) supplementation for 4 weeks failed to correct the reduced plasma vitamin A level in STZ-induced diabetic rats (Tuitoek et al. 1996a;Tuitoek et al. 1996c). Similar results have also been observed in diabetic BB rats. The depressed plasma vitamin A level and RBP concentration in diabetic BB rats could not be reversed by either vitamin A alone or a combination of zinc and vitamin A supplementation (Lu et al. 2000). It is, however, noteworthy that zinc supplementation significantly increased the hepatic RBP mRNA expression in BB rats in the presence of diabetes (Lu et al. 2000).

1.5 Objectives of present study

Our understanding of the depressed vitamin A metabolism in type 1 diabetes has progressed since this issue was first noticed in humans. However, there are still a lot of questions to be clarified in the relationship between vitamin A metabolism and type 1 diabetes. The alterations of retinol and RBP concentrations in plasma and liver were commonly examined in human and animal studies in the presence of diabetes (Tuitoek et al. 1996b;Tuitoek et al. 1996c;Lu et al. 2000). No data is available so far describing the vitamin A status in the pre-diabetic state. Such information is important in understanding the underlying causes of depressed vitamin A metabolic status in type 1 diabetes.

Plasma vitamin A levels are homeostatically regulated by several factors, one of which is retinyl ester hydrolysis. Retinyl ester hydrolysis participates in the intestinal uptake of dietary vitamin A, hepatic uptake of chylomicron remnant associated retinyl esters, mobilization of retinyl ester stores in the liver, and the use of vitamin A in extra-hepatic tissues. Despite these important roles, the relationship between retinyl ester hydrolysis and vitamin A status in type 1 diabetes has never been adequately studied.

Moreover, it is also important to explore the possible influence of dietary interventions such as vitamin A or zinc supplementation on retinyl ester hydrolysis if this process is seen to be altered in the presence of diabetes.

Thus, using BB rats as an animal model for type 1 diabetes, the present study was undertaken to test the following hypotheses:

1. There exists biochemical evidence of vitamin A deficiency prior to the onset of diabetes, independent of hyperglycemia;

2. The impaired vitamin A status at the onset of diabetes is related to the alteration of REH activities (such as bile salt-independent REH, CHAPS-stimulated REH and non-specific carboxylesterase) in both the intestine and the liver;

3. Decreased retinyl palmitate uptake by the jejunum or ileum may contribute to impaired vitamin A status at the onset of diabetes;

4. The REH activity is dose-dependently affected by dietary vitamin A intake in diabetic rats; vitamin A supplementation normalizes decreased plasma retinol level in these animals;

5. Dietary zinc supplementation improves REH activity and vitamin A metabolic status in diabetic rats; zinc also influences intestinal uptake of retinyl palmitate.

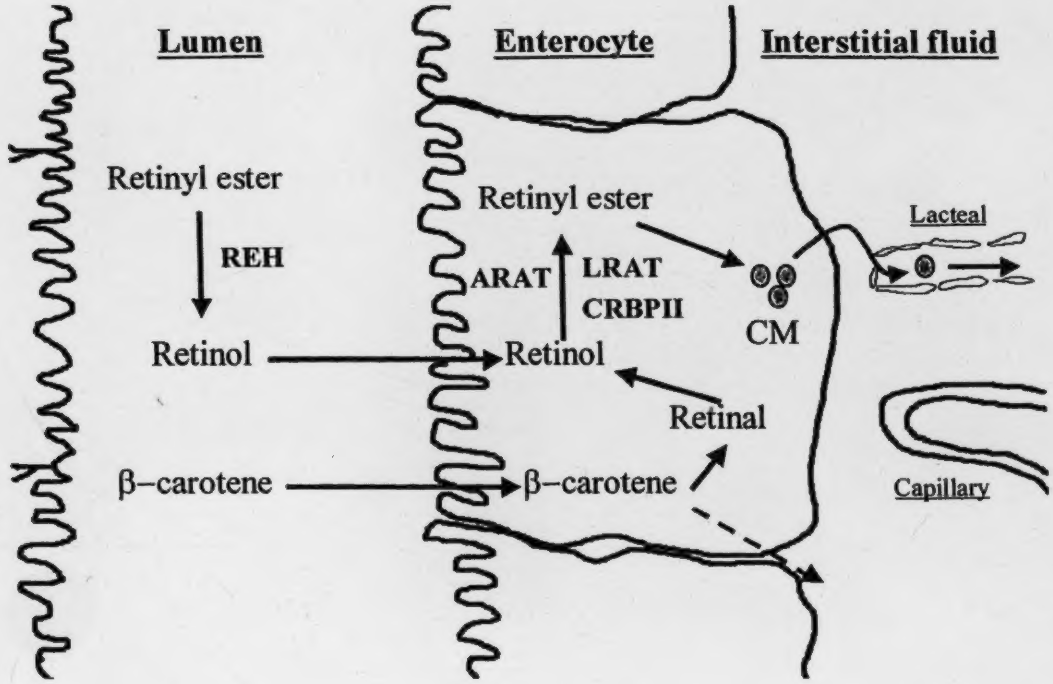

Figure 1-1 Overview of digestion and absorption of vitamin A in the small intestine.

Dietary retinyl esters are hydrolyzed in the lumen by enzyme retinyl ester hydrolase (REH) before the liberated retinol is taken up into the enterocytes. Meanwhile, β-carotene in the diet is absorbed into enterocytes through passive diffusion and converted to retinol through retinal. Under normal physiological condition, the newly absorbed free retinol in the enterocytes is bound to cellular retinol-binding protein II (CRBP II) and re-esterified with long-chain fatty acid by enzyme lecithin:retinol acyl-transferase (LRAT). When large amounts of retinol are digested, free retinol in the enterocytes is re-esterified by enzyme acyl-CoA:retinol acyltransferase (ARAT). The resulting retinyl esters are then packaged with other lipids into chylomicrons (CM) and transported via lymphatic system into the general circulation.

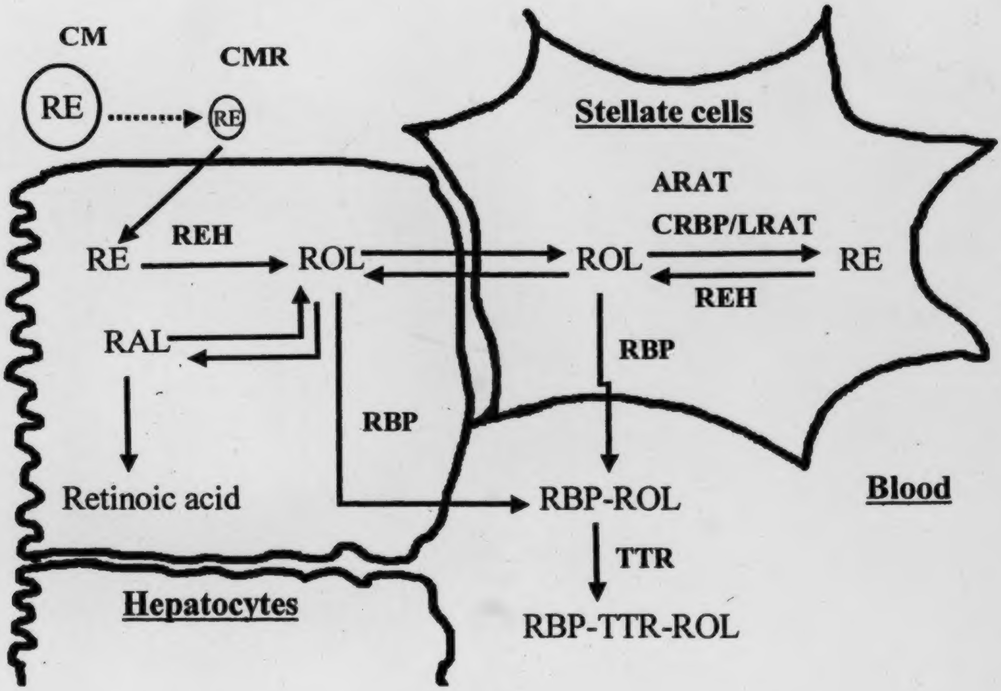

Figure 1-2 Overview of hepatic metabolism of vitamin A.

Most of the chylomicron remnant (CMR)-associated retinyl esters (RE) are rapidly taken up by hepatocytes and hydorlazed to retinol (ROL) through retinyl ester hydrolase (REH). Some of the liberated retinol is oxidized into retinal (RAL) and retinoic acid, or secreted into the general circulation bound with retinol-binding protein (RBP). The majority of retinol in hepatocytes is transferred into stellate cells, re-esterified to form RE and stored within stellate cells. Under normal physiological condition, free retinol bound with cellular retinol-binding protein (CRBP) in stellate cells is re-esterified via enzyme lecithin:retinol acyltransferase (LRAT). When retinol is present at high level, enzyme acyl CoA:retinol acyltransferase esterifies the excess retinol in its free form. Mobilization of retinol from its storage pools in stellate cells requires hydrolysis by REH. The freed retinol might be transferred back to hepatocytes, bound with RBP and secreted into the circulation. Alternatively, the free retinol might be secreted from stellate cells directly into the circulation bound with RBP. RBP-ROL complex in the blood is circulates with transthyretin (TTR) in a 1:1 molar ratio.

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Chapter 2

Age-associated change of vitamin A status in BB rats

2.1 Introduction

There appears to be considerable biochemical evidence regarding marginal vitamin A deficiency in type 1 diabetic patients. This is demonstrated by decreased levels of this vitamin in the plasma of such patients (Baena et al. 2002;Basu et al. 1989;Krempf et al. 1991;Krill et al. 1997;Merzouk et al. 2003;Martinoli et al. 1993;Granado et al. 1998). Similar results have been reported in STZ-induced diabetic rats and spontaneously developed diabetic BB rats (Tuitoek et al. 1996c;Lu et al. 2000). Those studies suggest that the decreased plasma retinol level in the presence of diabetes is secondary to the impaired transport of retinol from its hepatic storage pools, as evidenced by reduced circulatory levels of RBP or TTR in parallel with the lowered plasma retinol concentrations in diabetes (Tuitoek et al. 1996b;Basu et al. 1989;Lu et al. 2000).

The aforementioned human and animal studies were all carried out on diabetic subjects. The altered vitamin A metabolism reported in those studies appears to be related to the hyperglycemic state in the presence of diabetes. So far, not much is known about vitamin A status in pre-diabetic stages. This is a critical issue in order to better understand the metabolic derangement of vitamin A in type 1 diabetes. Such information is also particularly important in attempting to clarify the relationship between depressed vitamin A metabolic status, hyperglycemia and other metabolic alterations in the presence of diabetes. It was therefore hypothesized that biochemical evidence of marginal vitamin A deficiency might appear in rats in a normoglycemic state prior to the onset of diabetes. That hypothesis was tested using BB rats, one of the widely used animal models in studies of type 1 diabetes. In diabetes-prone BB rats (BBdp rats), about 40-80% of the animals develop diabetes with the peak incidence in the age range of 60 and 120 d (the period of adolescence/early adulthood in rats), and a median onset age of about 90 d in most reported data (Field and Butler 1999;Marliss et al. 1983). No difference in diabetes incidence exists between male and female BBdp rats (Nakhooda et al. 1977). In this study we examined the changes in plasma retinol levels in BBdp rats in the age range of 50 to 90 d at a 10 d interval as well as in diabetic BB rats (BBd rats) at the onset of diabetes. Non-diabetes-prone BB rats (BBn rats) were used as a control group in our study.

2.2 Materials and methods

Materials

The chemical reagents used were from Sigma-Aldrich Canada Limited (Oakville, Ontario, Canada) and Fisher Scientific Canada (Nepean, Ontario, Canada) unless otherwise stated.

Animals and diet

The animal protocol for the present study was reviewed and approved by the Faculty of Agricultural, Forestry and Home Economics Animal Policy and Welfare Committee, University of Alberta. The experiment was conducted in accordance with the Canadian Council on Animal Care Guidelines. BBdp rats and BBn rats as control were obtained from the breeding colony of the Department of Agricultural, Food and Nutritional Science, University of Alberta. The original breeding pairs were from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, Canada).

Sixteen weanling male BBdp rats and the same number of age-matched male BBn rats were housed individually in stainless steel, wire-bottom cages in a temperature and humidity controlled animal room with a 12-h light/dark cycle. The rats had free access to water and an open-formula, nonpurified NIH-07 basal diet in meal form (Ziegler Brothers, Gardner, Pennsylvania, USA, see Table 2-1 for dietary composition). The NIH-07 diet contained (g/kg) carbohydrate 514, fat 52, and protein 21.5, with the remaining weight accounted for moisture and non-metabolic solids. The vitamin A content of this diet was 2.61 mg RE/kg diet (added as retinyl acetate). This diet was chosen because it has a diabetogenic effects and routinely results in a high diabetes frequency in BBdp rats (Scott 1996), and thus would help us to observe the disease-related changes in the presence of diabetes.

During the experiment, food intake of each rat was recorded every other day, and body weight was monitored weekly. At the age of 50 d (pre-diabetic age), a 0.3 to 0.4 ml of blood was collected from tail vein of each BBdp or BBn rat in the morning (8-10 AM) after an over-night fast. Serum was separated from the blood sample and stored at –70°C for further analysis. Tail vein blood collection was repeated 10 d later and every 10 d thereafter until the rats reached the age of 90 d.

Once the BBdp rats were 50 to 52 d old, plastic containers were placed underneath a spout on the bottom of the cage to collect urine. BBdp rats were tested 3 times per wk for urinary glucose using Chemstrip uG (Boehringer Mannheim, Laval, Quebec, Canada) until the onset of diabetes, or until the rats were 120 d old at which time the experiment was ended. When the glucose levels in the urine of BBdp rats became 56 mmol/L or higher, a blood sample from the tail vein was taken in the fed state and glucose levels were measured using a glucometer (Ames Miles, Toronto, Ontario, Canada). The onset of diabetes was defined as the time when blood glucose concentrations became higher than 13.8 mmol/L in the fed state (Hosszufalusi et al. 1993). At the onset of diabetes BBdp rats were described as diabetic BB (BBd) rats. Each newly diagnosed BBd rat, and an age-matched BBn control rat, were terminated the the morning following the diabetes onset after an over-night fast. By the age of 120 d, all remaining BBdp rats (non-diabetes) were terminated after an over-night fast.

Blood and tissue samples collection

Before termination, the rats were anesthetized with 5% halothane (Halocarbon Laboratories, River Edge, New Jersey, USA). A sample of blood was collected via cardiac puncture and kept in a heparinized tube. The rats were then terminated via cervical dislocation. Later, plasma was separated by centrifugation at 800 g for 20 min at 4°C and stored at –70°C.

After rats were terminated, liver samples were quickly removed, rinsed with ice-cold saline, blotted, weighed, and cut into small pieces. The tissue was then quickly put into separate plastic vials and frozen immediately in liquid nitrogen before storage at –70°C for subsequent analysis.

Vitamin A and E determination

Plasma and hepatic free retinol, α-tocopherol, and hepatic retinyl palmitate were extracted from the samples without saponification. The analyses were done using reverse-phase high-performance liquid chromatography (HPLC) according to the methods of Tuitoek et al. (1996c), Wang et al. (1998) and Vliet (1991) with minor modifications. Liver samples were also saponified and analyzed by reverse-phase HPLC for total hepatic vitamin A concentrations (as retinol) by the method of Ross (1986) with modification.

- Sample preparation

To extract free retinol from plasma samples, a 200 μl plasma sample was vortex-mixed in a 1.5 ml micro-centrifuge tube with 20 μl of an internal standard (retinyl acetate in acetonitrile) and 80 μl of acetonitrile as precipitating agent. The mixture was extracted with 200 μl of butanol: ethyl acetate (1:1, v/v) mixture and vortex-mixed for one min. A 100 µl aqueous solution of dipotassium monohydrogen phosphate (1.2 g/ml) was then added and vortex-mixed for 30 seconds. After centrifugation for 2 min at 10,000 g to remove precipitated proteins, the organic layer was transferred to an HPLC vial for analysis.

The liver tissue (100 mg) was homogenized with 0.5 ml of ice-cold phosphate buffered saline (PBS) (0.05 mol/L, pH 7.4) using a Polytron homogenizer (Brinkmann, Westbury, New York, USA). After homogenization, the liver sample was mixed with an internal standard (retinyl acetate in methanol) and 0.5 ml of acetonitrile to precipitate proteins, and then extracted with 0.5 ml of butanol: ethyl acetate (1:1, v/v) mixture. A 200 µl aqueous solution of dipotassium monohydrogen phosphate (1.2 g/ml) was then added and vortex-mixed. The mixture was centrifuged for 10 min at 3000 rpm and the organic layer was transferred to a HPLC vial for analysis for free retinol and retinyl palmitate in the liver.

For the analysis of hepatic total vitamin A (as retinol), a 1 g liver sample was homogenized with 2 ml of ice-cold PBS buffer (0.05 mol/L, pH 7.4) using a Polytron homogenizer. The liver homogenate (0.1 ml) was then saponified in a freshly prepared solution of 95% methanol/5% potassium hydroxide containing 1% (w/v) pyrogallol as antioxidant at 70°C for 30 min. After saponification, the mixture was cooled in an ice bath and then extracted twice with hexane (2 ml) followed by 1 ml of water. After centrifugation for 5 min at 2500 rpm, a measured portion (about 1.5 ml) of the upper phase was transferred into a vial and an appropriate amount of retinyl acetate was added as internal standard. Solvent in each vial was evaporated to dryness under nitrogen at 37-40°C. The residue was redissolved completely in 150 µl methanol and was then ready for injection into the HPLC system.

- Standard solutions

Retinol, retinyl palmitate and α-tocopherol standard stock solutions (15 mg of retinol and α-tocopherol, and 25 mg of retinyl palmitate, respectively) and retinyl acetate internal standard stock solution (15 mg of retinyl acetate) were each prepared in 10 ml of ethanol. The working solutions were prepared from the stock solutions by diluting the stock solutions in a suitable amount of acetonitrile. The standard stock solutions were stored at -20°C, protected from light, and used within one month of preparation.

The concentrations of the standard stock solutions were determined by measuring the absorbance of diluted stock solutions using a spectrophotometer (Cary UV-Visible spectrophotometer, Varian Instruments, Sugar Land, Texas, USA) every time before the standard solutions were used. The absorbance was monitored at 325 nm for retinoids and 292 nm for α-tocopherol. The concentrations of standard stock solutions were calculated by using the standard absorbance $E^{1\%}_{1cm}$: retinol 1845, retinyl palmitate 940, α-tocopherol 84, and retinyl acetate 1560 (Barua and Furr 1998;Hess et al. 1990).

- Chromatography

A 50-μl aliquot of each extract from plasma and liver samples was injected into a Varian 5000 reverse phase HPLC system (Varian Canada Ltd., Mississauga, Ontario, Canada) with a 5 μm Supelcosil LC-18 column (0.46 × 15 cm; Supelco, Ontario, Canada). The running column was preceded by a C_{18} guard column. A Waters-486 tunable absorbance detector (Waters Chromatography Division, Millipore Milford, Massachusetts, USA) was set at 325 nm to identify retinol and retinyl esters. A Shimadza RF-535 fluorescence HPLC monitor (Shimadza Corporation, Japan) provided excitation at 295 nm and emission at 330 nm was used to detect α-tocopherol.

The mobile phase used for the plasma samples was methanol-water (95: 5, v/v) at a flow rate of 1.5 ml/min. Retention time for retinol was about 3.2 min and for α-tocopherol was about 10.2 min. The mobile phase used to analyze hepatic total vitamin A (as retinol) in saponified liver samples was methanol-water (90: 10, v/v) at a flow rate of 2 ml/min. Retention time for retinol was about 3.4 min. The mobile phase used for hepatic free retinol, retinyl palmitate and α-tocopherol in liver samples without saponification consisted of acetonitrile-tetrahydrofuran-water (solvent A: 50:20:30; solvent B: 50:44:6, v/v/v, with 1% (w/v) ammonium acetate and 0.35% (v/v) acetic acid in water) at a flow rate of 1 ml/min. The gradient procedure for the latter mobile phase was 100% solvent A for 6 min followed by a 6-min linear gradient to 60% solvent A and 40% solvent B, a 6-min gradient to 100% solvent B, a 5-min hold at 100% solvent B, a one-min back to 100% solvent A, and then a 6-min hold at 100% solvent A. Retention time for retinol was 7.9 min and for retinyl palmitate was 19.8 min. Retention time for α-tocopherol was 17 min. All runs were performed at ambient temperature.

Retinol, retinyl palmitate and α-tocopherol were identified by comparison of retention times with those of standards injected separately onto the same column, and also by spiking sample extracts with the different standards. Each retinol or retinyl palmitate/internal standard (retinyl acetate) peak-area ratio was compared to the calibration curve to quantify retinol or retinyl palmitate. Alpha-tocopherol concentrations in the sample were calculated using an external standard.

Glucose and lipids analysis

Plasma glucose levels were measured by the glucose oxidase method using a Sigma kit (Procedure No. 315). Plasma triglyceride (TG) and total cholesterol (TC) levels were measured using enzymatic kits from Sigma (Procedure No. 336 and 401). Plasma high density lipoprotein (HDL)-cholesterol was determined using the same kit for total cholesterol measurement in the supernatant of plasma sample after very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated using Sigma HDL cholesterol reagent (Catalog No. 352-4).

Hepatic triglyceride and total cholesterol were measured by the enzymatic methods adapted from Carr et al.(1993) and Omodeo-Salè et al.(1984). Liver samples (50 mg) were added to 0.6 ml of distilled water and homogenized using a Polytron homogenizer. Two ml of hexane:iso-propanol (3:2, v/v) was then added and the mixture was vortex-mixed for 2 min to extract tissue lipids according to the method of Radin (1981). An appropriate aliquot of sample extract (about 1 ml) was pipetted into a new screw-cap tube. Half a ml of Triton X-100 solution in iso-propanol (2%, v/v) was added to each tube and vortex-mixed. Distilled water (0.5 ml) was added to each tube after the solvent in the tube was dried under nitrogen at 45°C and then cooled to room temperature. The tubes were capped and placed in a shaking water bath for 15 min at 37°C to solubilize the lipids. Cholesterol standards were made in different concentrations in iso-propanol and solubilized following the same procedure as that for sample preparation.

After lipid solubilization, the level of triglyceride in the liver was determined by using the enzymatic kit from Sigma (Procedure No. 336). The assay for total cholesterol in the liver was performed by adding an aliquot of sample or standard to an enzyme mixture (every ml of enzyme mixture contained 0.25 U of cholesterol oxidase, 0.5 U of cholesterol ester hydrolase and 1 U of horseradish peroxide). After the samples and enzyme mixture were mixed and allowed to stand at room temperature for 30 min, the absorbance was measured at 500 nm vs a reagent blank. The same procedure described for total cholesterol was followed for the analysis of the free cholesterol level in the liver after cholesterol ester hydrolase was excluded from the enzyme mixture.

All assays were performed in 96-well microtiter plates and the plates were read on a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA).

Statistical analysis

All statistical analyses were conducted using the SAS system (SAS Institute, Cary, North Carolina, USA). Results were expressed as mean values with their standard errors (SEM). Prior to the onset of diabetes, the main effects of age (50 to 90 d, at 10-d intervals) and disease (diabetes-prone, and non-diabetes-prone) and their interaction were assessed with a two-way analysis of variance (ANOVA). At the onset of diabetes, BBd rats were compared using a one-way ANOVA with age-matched BBn rats as well as BBdp rats aged 120 d. Differences between groups were identified by least squares means. A two-tailed *P*-value of ≤ 0.05 was considered significant.

2.3 Results

Prior to the onset of diabetes

Results in Figure 2-1 show the change of daily food intake and body weight gain in BB rats prior to the onset of diabetes. During the ages of 50 to 90 d, there was no significant difference in daily food intake or body weight gain between BBdp and BBn rats.

During the pre-diabetic stages, plasma glucose levels were significantly lower in BBdp rats compared to that of the BBn rats at the ages of 50, 60 and 70 d. However, the difference disappeared when the rats were older. No differences in the plasma glucose levels were observed between BBdp and BBn rats at the age of 80 and 90 d (Table 2-2).

Plasma triglyceride levels in BBdp rats were similar to those of BBn rats except at the ages of 70 and 90 d, at which points decreased plasma triglyceride levels were observed in BBdp rats (Figure 2-2 A). Plasma total cholesterol levels were consistently higher in BBdp rats compared to that of BBn rats from the age of 60 to 90 d. However no difference was observed between the groups at the age of 50 d (Figure 2-2 B).

Compared to BBn control rats of the same age, consistently lower plasma retinol levels were observed in BBdp rats at the ages of 50, 60, 70 and 80 d (Figure 2-3). However, the difference in plasma retinol levels was not significant in the rats of 90 d old.

Contrary to the changes in plasma retinol levels, plasma α-tocopherol levels in BBdp rats before the onset of diabetes were significantly higher than that of BBn rats at all ages of 50 to 90 d (Figure 2-4 A). Similar results were obtained in lipids-corrected α-tocopherol level (α-tocopherol: lipids ratio) in plasma at the same age range (Figure 2-4 B).

At the onset of diabetes

By the end of the experiment, six out of sixteen BBdp rats showed symptoms of glycosuria and hyperglycemia and were thus diagnosed as diabetic rats. Those rats were terminated on the second day of diabetes onset along with the age-matched BBn control rats. The earliest onset age among diabetic BB rats was 61 d, and the latest was 113 d, with an average onset age at 95 d. The cumulative diabetic incidence in BBdp rats was 37.5% by the age of 120 d.

Results from diabetic BB rats at the onset of diabetes were compared with age-matched BBn control rats, and BBdp rats that failed to show signs of diabetes before the age of 120 d. Neither the final body weight nor the daily food intake was found to be significantly different between age-matched diabetic rats and control animals (Table 2-3). Body weight in BBdp rats at 120 d was higher than that of BBd rats, while no difference was found between BBdp and BBn rats. Liver weight was similar among all three groups of rats. Significantly higher blood glucose levels and 24 h urine excretions were detected in diabetic rats compared with that of age-matched BBn rats and BBdp rats at the age of 120 d.

At the onset of diabetes, diabetic BB rats had significantly higher plasma total cholesterol levels and plasma triglyceride levels than age-matched BBn controls, while plasma HDL-cholesterol levels did not differ between BBd and BBn rats (Table 2-4). BBdp rats aged 120 d had similar plasma triglyceride levels than BBd and BBn rats. The total cholesterol levels in these BBdp rats were similar to those of diabetic BB rats but they were higher than that of the non-diabetic control rats. HDL-cholesterol levels in BBdp rats were similar to both diabetic BBd and non-diabetic BBn rats.

Hepatic triglyceride levels did not differ among the BBd, BBn and BBdp rats. However, BBdp and BBn rats showed the lowest and highest hepatic total and free cholesterol levels, respectively, among the three groups of rats. Diabetic BB rats had significantly lower levels of hepatic total and free cholesterol than age-matched BBn controls (Table 2-4).

Plasma free retinol levels in diabetic BB rats were significantly lower than that in non-diabetic control rats. BBdp rats aged 120 d had intermediate plasma retinol levels to both diabetic BB rats and non-diabetic control animals (Figure 2-5).

Similar to the changes in plasma retinol, hepatic free retinol levels were significantly lower in diabetic BB rats at the onset of diabetes than that of age-matched BBn control animals. BBdp rats aged 120 d also had lower free retinol levels in the liver than BBn rats. No difference in hepatic free retinol levels were observed between BBd rats at the onset of diabetes and BBdp rats aged 120 d (Figure 2-6 A). Hepatic retinyl palmitate concentrations and total vitamin A levels (as retinol) did not differ significantly among diabetic BB rats and age-matched BBn controls, and BBdp rats aged 120 d (Figure 2-6 B). The ratios of hepatic retinyl palmitate to free retinol in both diabetic rats and diabetes-prone rats were markedly higher than that in the non-diabetes BB controls, whereas no difference in the ratios were found between BBd and BBdp rats (17.24±2.21, 14.93±2.05 and 10.87±0.91 for BBd, BBdp and BBn rats, respectively; BBd or BBdp *vs* BBn, $P<0.05$; BBd *vs* BBdp, $P>0.05$).

Both plasma and hepatic α-tocopherol levels were significantly different among BBd rats, their age-matched BBn control, and BBdp rats aged 120 d. BBd rats had the highest plasma and hepatic α-tocopherol levels, whereas the levels in BBn rats were the lowest among the three groups of rats. Plasma α-tocopherol levels in BBdp rats were higher than that of BBn rats, but lower than that of BBd rats (Figure 2-7 A). No significant difference in hepatic α-tocopherol levels was found between BBdp and BBd rats (Figure 2-8). Lipids-corrected α-tocopherol levels in plasma showed similar differences between groups as plasma α-tocopherol levels. The ratios of plasma α-tocopherol to lipids were significantly higher in BBd rats than that in age-matched BBn rats (Figure 2-7 B). The ratios were markedly lower in BBdp rats aged 120 d than that of BBd rats, but did not differ from BBn rats.

2.4 Discussion

Decreased plasma retinol levels have been reported in type 1 diabetic patients (Baena et al. 2002;Basu et al. 1989), STZ-induced diabetic rats (Tuitoek et al. 1996c), and spontaneously developed diabetic BB rats (Lu et al. 2000). So far, little is known about the vitamin A status in the pre-diabetic state. In this study, changes in plasma retinol, α-tocopherol, lipids and glucose levels were monitored in diabetic-prone BB rats and non diabetic-prone BB control rats at ages of 50 to 90 d. The relevant biochemical indices in BB rats were examined during this period because most diabetes-prone BB rats develop the disease during the age period of 60 to120 d with a median of about 90 d. Thus, monitoring the biochemical changes of rats during these ages will allow an understanding of vitamin A status at the pre-onset stages in diabetic BB rats.

Results from this study clearly demonstrate that the plasma retinol levels were altered in BBdp rats prior to the onset of diabetes. BBdp rats had markedly decreased plasma retinol levels compared to age-matched BBn control during the age range of 50 to 80 d. However, such differences were not significant at the age of 90 d. Results also showed that plasma retinol levels in diabetic BB rats were significantly decreased at the onset of diabetes compared with their age-matched BBn controls, which is in agreement with the results from a previous study (Lu et al. 2000). These results confirm that vitamin A metabolic status is depressed at the onset of diabetes in BB rats and further indicates that altered vitamin A status appears not only in diabetic BB rats, but also in BBdp rats prior to the onset of diabetes when plasma glucose is still within the normal range.

Several possible mechanisms have been suggested for the decreased plasma retinol level in type 1 diabetes. These include: lowered conversion of β-carotene to vitamin A (Gouterman and Sibrack 1980;Krill et al. 1997); poor glycemic control (Schultz et al. 2001); increased consumption of retinol resulting from the process of eliminating oxygen free radicals in diabetes (Martinoli et al. 1993); insulin deficiency (Tuitoek et al. 1996a;Basu and Basualdo 1997); reduced vitamin A carrier proteins including RBP and TTR (Tuitoek et al. 1996b;Basu et al. 1989;Lu et al. 2000); and increased urinary zinc loss as well as decreased total body zinc levels (Christian and West 1998;Lu et al. 2000). Moreover, a genetic component is thought to play a role in the reduced expression of RBP thus contributing to the decreased plasma retinol level in diabetic patients (Krill et al. 1997).

In the present study, the decreased plasma retinol levels observed in BBdp rats prior to the onset of diabetes, and in diabetic BB rats, are not likely to be explained by dietary vitamin A intake because the daily food intake as well as body weight gain in BBdp rats were similar when compared with control animals. This conclusion is supported by a previous study in which the plasma retinol levels remained low in pair-fed diabetic rats induced by STZ (Tuitoek et al.

1996c). Furthermore, the intestinal absorption of retinol in STZ-induced diabetic rats was unaffected by the presence of diabetes (Tuitoek et al. 1994).

Results from our study demonstrate that decreased plasma retinol levels are not directly related to the hyperglycemic state in BB rats. Not only diabetic BB rats with high blood glucose levels had lower plasma retinol concentrations at the onset of diabetes, normal glycemic BBdp rats at the pre-diabetic stage also had depressed plasma retinol levels compared to control animals. These results are in agreement with another study, in which no relationship was found between plasma vitamin A and glycemic control, estimated by glycosylated hemoglobin (HbA_1c) despite significantly decreased plasma vitamin A concentrations in adult type 1 diabetic patients (Krempf et al. 1991). Meanwhile, no difference in plasma vitamin A concentration was found between children with good and poor control of their illness based on the HbA_1C levels (Baena et al. 2002). These results suggest that decreased plasma retinol levels may not be associated with hyperglycemia in type 1 diabetes.

It is noteworthy that reduced plasma retinol levels are associated with type 1 diabetes when there is a lack of insulin production, but not with type 2 diabetes accompanied by insulin resistance (Krempf et al. 1991;Basualdo et al. 1997;Abahusain et al. 1999). In the type 1 diabetes animal model, BB rats, the onset of diabetes is generally rapid and severe. The secretion of insulin in response to glucose is severely blunted at the onset of diabetes although the islets from diabetic BB rats still have 60% of insulin level compared with that of control animals within 12 hour of diabetes onset (Pederson et al. 1990). BBd rats one day after the diagnosis of diabetes demonstrate fasting hyperglycemia as well as glucose intolerance. The insulin response to glucose in these diabetic BB rats is decreased by 95% (Pederson et al. 1990). The pancreas of overt diabetic BB rats usually has less than 0.1% of normal insulin content and immunoreactive insulin declines to immeasurable values within four to eight d of diabetes onset (Marliss et al. 1983). In addition to these changes at the onset of diabetes, the decreased insulin release in response to glucose has also been observed in pre-diabetic BB rats. The total amount of insulin-release during glucose stimulation from diabetic-prone BB rats aged 50 d was reduced by almost 50% compared to age-matched non-diabetic BB control rats (Svenningsen et al. 1986). The extractable pancreatic insulin was 30% less in the diabetic-prone BB rats (Svenningsen et al. 1986). In the present study, it was found that the plasma retinol levels were decreased not only at the onset of diabetes, but also in pre-diabetic BB rats compared with the control animals. It is thus possible that the reduced plasma retinol levels in pre- and post-diabetic BB rats are, at least in part, related to the decreased insulin secretion and/or pancreatic insulin content. Indeed, insulin treatment for four weeks has been shown to restore a decreased plasma vitamin A level to its control level in STZ-induced diabetic rat (Tuitoek et al. 1996a).

A relationship between insulin and vitamin A is also evidenced by the fact that vitamin A deficiency affects insulin secretion, whereas insulin secretion can be restored to a normal level by additional dietary vitamin A (as retinol or retinoic acid) in rats (Chertow et al. 1987). Retinol and retinoic acid have also been reported to increase an *in vitro* release of insulin in β cells of the rat insulinoma line (RINm5F) (Chertow et al. 1989). These results suggest that vitamin A is required for insulin release. The influence of vitamin A on insulin release is proposed to be mediated by transglutaminase activity in islets (Driscoll et al. 1997). Considering together, the reduced insulin secretion and/or pancreatic insulin content in BB rats pre- and post-diabetes may thus account for the reduced plasma retinol levels observed in these animals. On the other hand, the reduced plasma retinol level in these animals may, in turn, affect insulin secretion. The modifying effect of insulin on vitamin A status in the presence of diabetes is not supported by a recent clinical study (Granado et al. 2003). Following an intensive insulin therapy for 3 to 33 months, the type 1 diabetic patients in that study were able to bring down their glycemic index to the normal level, while the decreased plasma retinol levels in these patients remained unaffected.

Lu et al (2000) suggested that depressed vitamin A status in the presence of diabetes is probably related to the altered transport of retinol from its hepatic stores, as evidenced by decreased plasma levels of RBP as well as the hepatic RBP gene expression in diabetic rats. The present study further demonstrates that in parallel with the decreased plasma retinol levels, the hepatic free retinol levels were significantly decreased, whereas the ratios of retinyl palmitate to free retinol were increased in diabetic BB rats. No significant changes in hepatic total vitamin A or retinyl palmitate levels were observed in diabetic rats. These results suggest the possibility that retinyl ester hydrolysis contributes to the deranged retinol mobilization in the liver. The liberated retinol, following retinyl ester hydrolysis from its hepatic storage pools, becomes metabolically available for transport into the circulation bound with its carrier protein, RBP (Blaner and Olson 1994). The hepatic hydrolysis of retinyl ester is thus a determining factor for retinol availability in the liver. It seems possible that the decreased circulatory levels of retinol in diabetic rats may be a reflection of the decreased metabolic transfer of retinyl ester to liberated retinol in the liver.

It is noteworthy that in parallel with the decreased plasma retinol levels, the plasma cholesterol concentrations were consistently elevated in BBdp rats prior to the onset of diabetes. These changes may also be due to an altered insulin level in the pre-diabetic state. A low insulin level in type 1 diabetes increases the activities of two important enzymes in reverse cholesterol transport, including cholesterol ester transfer protein, and lecithin:cholesterol acyltransferase (LCAT), and thus leads to an accumulation of LDL-cholesterol ester in the circulation (Chang et al. 2001). This influence of insulin on cholesterol metabolism probably explains the increased plasma cholesterol level in our study. Consistent with the lipid changes in the pre-diabetic state, dyslipidemia was observed in diabetic BB rats, as evidenced by increased plasma cholesterol and triglyceride levels in these animals. No differences in HDL-cholesterol concentrations were found in diabetic rats. Meanwhile, hepatic total cholesterol concentration was significantly decreased in diabetes. It is generally reported that insulin deficiency reduces lipoprotein lipase activity and results in defective removal of triglyceride-rich lipoproteins, including chylomicrons, chylomicron remnants and VLDL, and therefore is associated with an increased level of triglyceride (Brunzell et al. 1979;Yoshino et al. 1996). A higher level of triglyceride may influence HDL metabolism by promoting more cholesterol esterification and more esterified cholesterol enrichment in apolipoprotein B-containing lipoprotein such as VLDL, intermediate density lipoprotein (IDL) and LDL (Valabhji et al. 2002;Ritter and Bagdade 1996). This may eventually lead to a normal or even higher HDL-cholesterol level in type 1 diabetes. These lipids abnormalities are likely to play an important role in the subsequent development of coronary heart disease and other complications in diabetes.

In parallel with the alterations of plasma cholesterol concentrations, plasma α-tocopherol levels as well as lipid-corrected α-tocopherol concentrations were significantly elevated in BBd rats at the onset of diabetes. This result is in agreement with some studies (Behrens et al. 1984;Sun et al. 1999;Vessby et al. 2002) but not others (Young et al. 1995;Young et al. 1992). Consistently, plasma α-tocopherol levels were augmented in BBdp rats compared to their counterparts in the pre-diabetic stage. Hepatic α-tocopherol levels were also increased in the presence of diabetes. The similarity of changes in vitamin E and lipids in pre-diabetic rats, as well as diabetic rats, may be explained by the close association between vitamin E and lipids in metabolic pathways. Briefly, vitamin E in the diet is absorbed in the intestine along with other dietary lipids including triglyceride, cholesterol and vitamin A. These lipids are packaged into chylomicrons and secreted into the circulation via the lymphatic pathway. During chylomicron catabolism, some of the vitamin E is distributed to other circulating lipoproteins and it is ultimately taken up by extrahepatic tissues. The major portion of vitamin E in chylomicron remnants is taken up by the liver. By the action of α-tocopherol transport protein in the liver, newly absorbed vitamin E is incorporated into nascent VLDL with triglyceride, cholesterol, phospholipids, and apolipoproteins. The VLDL is then secreted into the circulation, where vitamin E is then transported by lipoproteins in the plasma along with other lipids and delivered to tissues by the action of lipoprotein lipase and corresponding receptors (Traber and Sies 1996;Herrera and Barbas 2001). It has been reported that the delivery of vitamin E is simultaneously accompanied by the delivery of cholesteryl linoleate (Traber and Sies 1996). Thus, the elevation of plasma and hepatic vitamin E levels in diabetic BB rats may be partly explained by lipid disorders in these rats. Additionally, it is also suggested that the increased level of plasma vitamin E is related to a defect in vitamin E transport into cells (Caye-Vaugien et al. 1990), poor control of glycemia (Campoy et al. 2003), or insulin deficiency (Behrens et al. 1984) in the presence of diabetes. A study by Behrens found that the treatment of diabetic rats with insulin for 30 d enabled return of the increased plasma and tissue vitamin E concentrations back to the levels of control animals (1984). The importance of altered vitamin E status in type 1 diabetes or the related mechanism(s) are still not clear. However, the beneficial effect of vitamin E as an antioxidant on oxidative stress in the pathogenesis of diabetes as well as its complications has long been emphasized (Jialal et al. 2002;Rosen et al. 2001;Opara 2002).

Table 2-1 Composition of NIH-07 diet [†]

Ingredient	Amount (g/kg diet)
Ground No.2 yellow shelled corn	243
Ground hard winter wheat	230
Soybean meal	120
Wheat middlings	100
Fish meal	100
Dried skim milk	50
Alfalfa meal	40
Corn gluten meal	30
Soybean oil	25
Brewers dried yeast	20
Dry molasses	15
Dicalcium phosphate	12.5
Ground limestone	5
Sodium chloride	5
NIH-07 vitamin premix [1]	2.5
NIH-07 mineral premix [2]	1.1
Choline chloride	1

† Data provided by diet supplier Zeigler Brothers, Inc. (Gardners, Pennsylvania, USA)

1. Vitamin premix contains (/kg diet): vitamin A (vitamin A acetate) 6057 IU (or, 1.8 mg RE), vitamin D_3 5066 IU, vitamin E (dl-α-tocopheryl acetate) 22 IU, vitamin K (menadione sodium bisulfite) 3 mg, thiamin 11 mg, riboflavin 37 mg, niacin 33 mg, d-pantothenic acid (calcium pantothenate) 20 mg, pyridoxine 6.5 mg, d-biotin 0.15 mg, folic acid 2.4 mg, vitamin B_{12} 50 μg
2. Mineral premix contains (/kg diet): manganese 66 mg, zinc 17.6 mg, iron 132 mg, copper 4.4 mg, iodine 1.5 mg, cobalt 440 μg

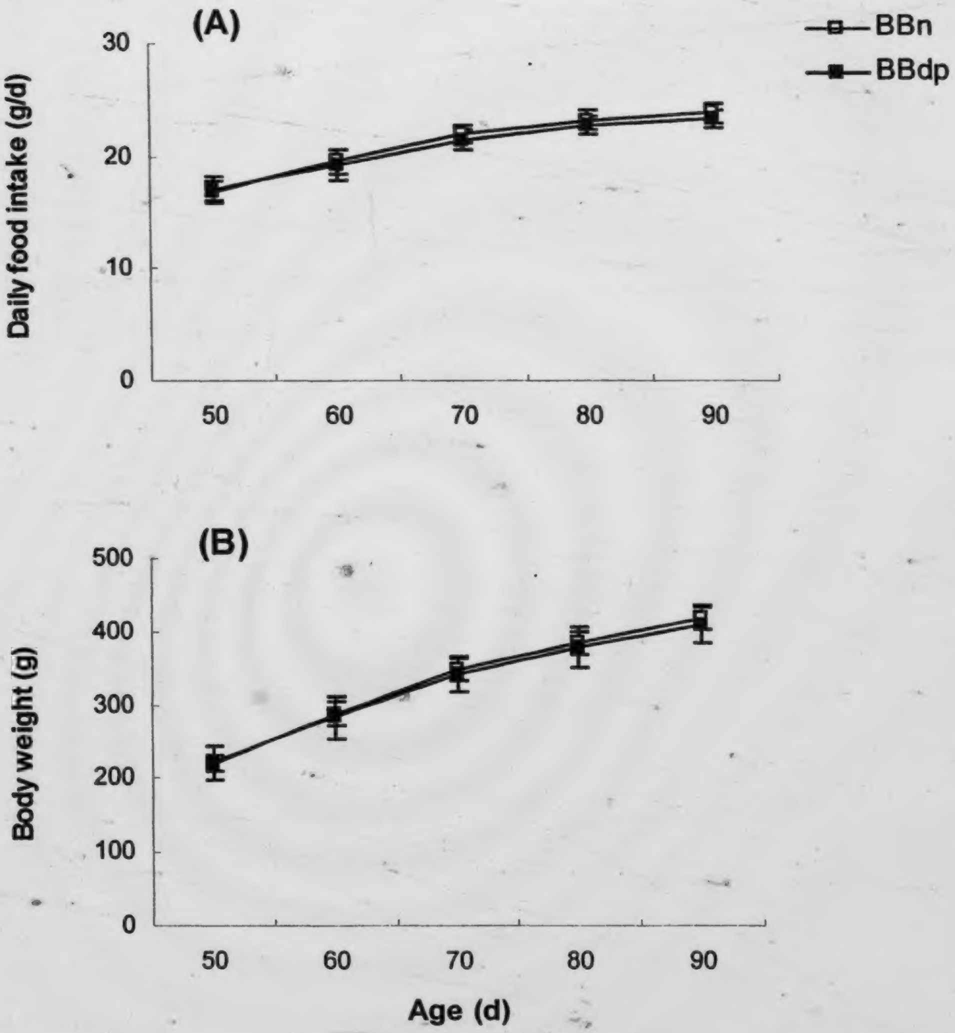

Figure 2-1 Daily food intake (A) and body weight gains (B) in BBdp and BBn rats from the age of 50 to 90 d.

BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to sixteen rats with their standard errors shown by vertical bars. No significant difference was detected in either daily food intake or body weight gains between BBdp and BBn rats during this period.

Table 2-2 Plasma glucose levels (mmol/L) in BBdp and BBn rats from the age of 50 to 90 d[†]

(Mean values with their standard errors (SEM) for six to sixteen rats per group)

Age (d)	BBn		BBdp		Significance, P*
	Mean	SEM	Mean	SEM	
50	5.23^{a}	0.08	4.04^{a}	0.18	0.0002
60	5.39^{a}	0.28	4.28^{a}	0.09	0.0035
70	6.67^{b}	0.32	4.94^{b}	0.19	0.0001
80	5.94^{ab}	0.49	5.35^{b}	0.24	NS
90	6.77^{b}	0.32	6.16^{c}	0.18	NS

†. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Blood samples were taken via tail breeding in BBdp and BBn rats from the age of 50 to 90 d at a 10-d interval.

* Significant difference between BBdp rats and age-matched normal BBn controls. NS = $P>0.05$.

Mean values that do not share a common superscript within a column are significantly different as determined by two-way ANOVA with least squares means test ($P<0.05$).

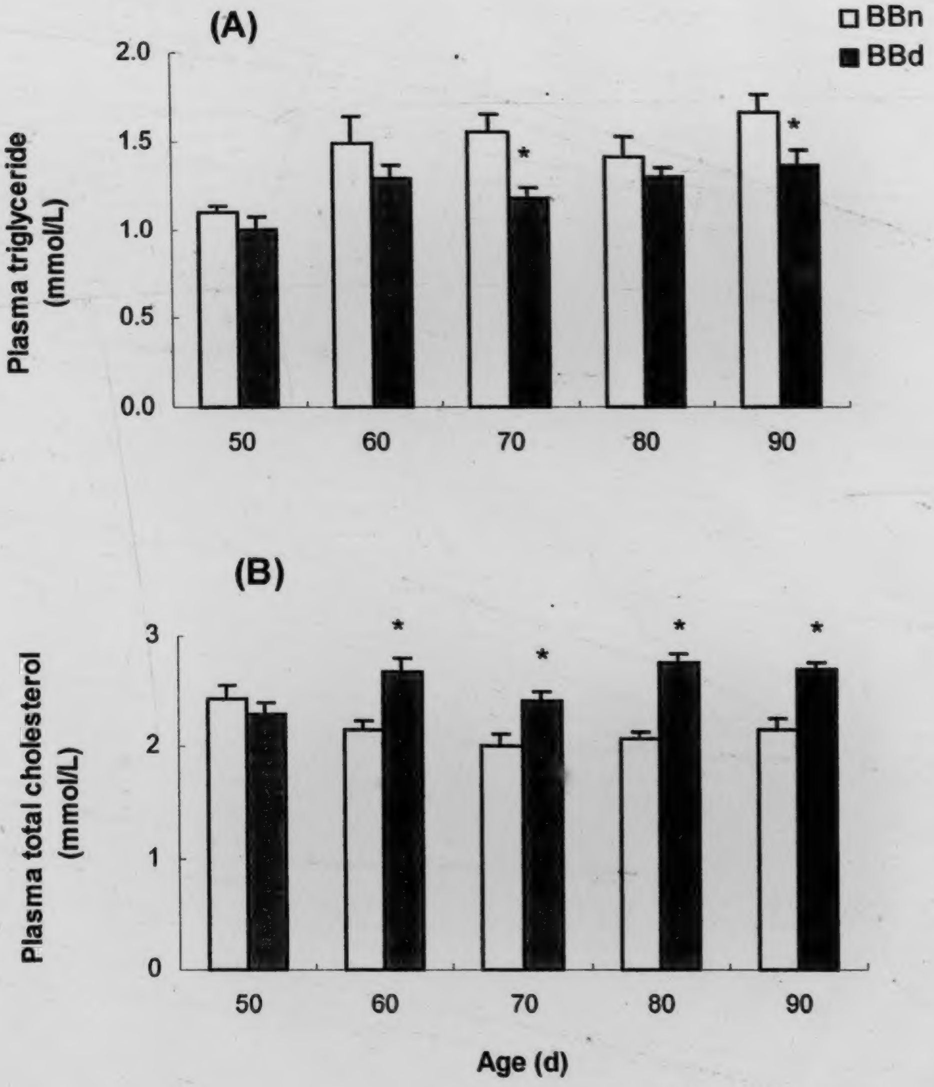

Figure 2-2 Plasma triglyceride (A) and total cholesterol (B) levels in BBdp and BBn rats from the age of 50 to 90 d.

BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to sixteen rats with their standard errors shown by vertical bars. * indicates a significant difference between BBdp and age-matched BBn rats ($P<0.05$).

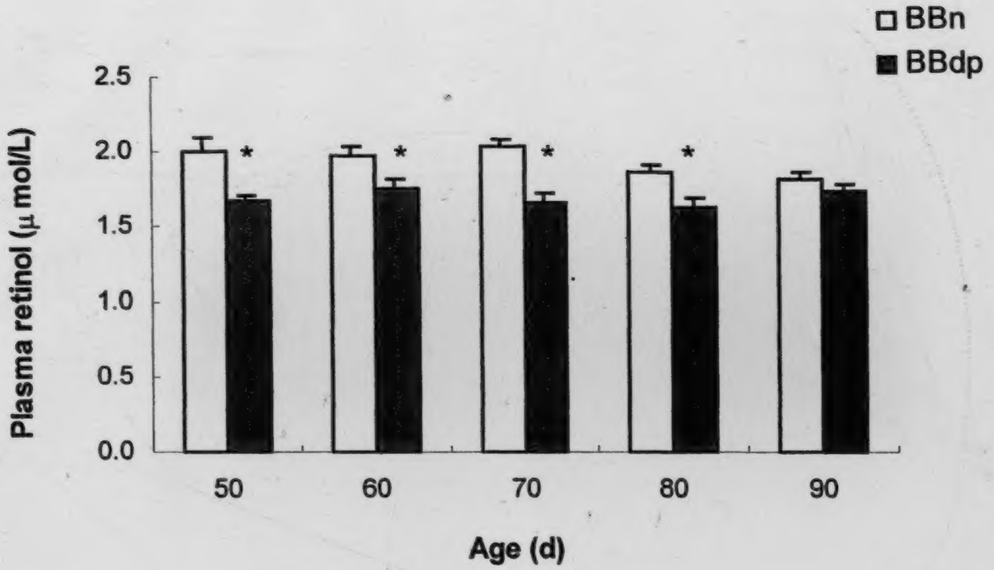

Figure 2-3 Plasma retinol levels in BBdp and BBn rats from the age of 50 to 90 d.

BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to sixteen rats with their standard errors shown by vertical bars. * indicates a significant difference between BBdp and age-matched BBn rats ($P<0.05$).

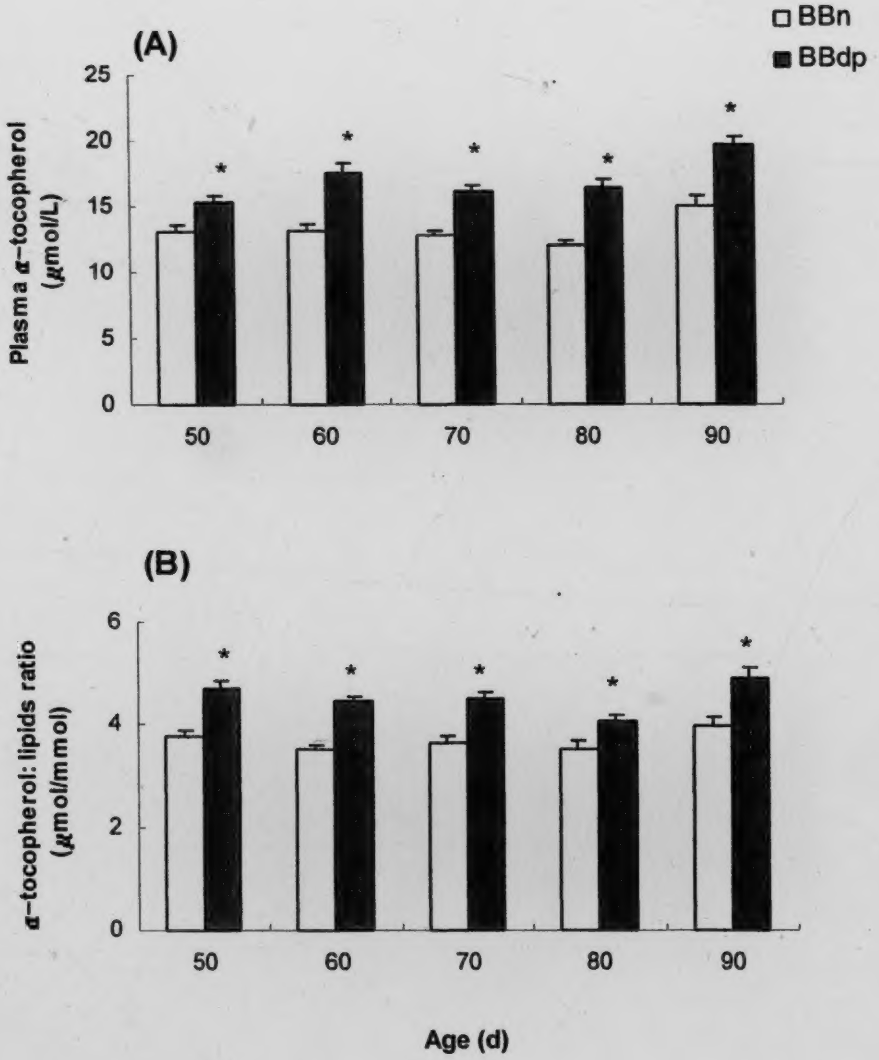

Figure 2-4 Plasma α-tocopherol levels (A) and plasma α-tocopherol: lipids ratios (B) in BBdp and BBn rats from the age of 50 to 90 d.

BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Plasma lipids are the sum of plasma triglyceride and total cholesterol. Values are means of six to sixteen rats with their standard errors shown by vertical bars. * indicates a significant difference between BBdp and age-matched BBn rats ($P<0.05$).

Table 2-3 General characteristics and plasma glucose levels in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d [†]

(Mean values with their standard errors (SEM) for six to ten rats per group)

	BBn (*n* 6)		BBd (*n* 6)		BBdp (*n* 10)	
	Mean	SEM	Mean	SEM	Mean	SEM
Food intake (g/d)	22.60^{a}	0.99	21.52^{a}	1.19	23.25^{a}	0.33
Final body weight (g)	423.51^{ab}	31.69	395.83^{a}	43.19	476.8^{b}	12.45
Liver weight (g)	13.79^{a}	0.85	14.33^{a}	1.81	14.68^{a}	0.64
24 h urine volume (ml/24 h)	4.87^{a}	0.85	60.20^{b}	13.02	8.97^{a}	1.22
Plasma glucose (mmol/L)	6.29^{a}	0.37	15.66^{b}	2.46	8.71^{a}	0.62

[†] BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. BBd and age-matched BBn rats were killed at the time of diabetes onset. The average onset age of diabetes was 95.3 ± 7.4 d with the earliest at 61 d and the latest at 113 d. BBdp rats were killed at the age of 120 d when the experiment was terminated.

Mean values that do not share a common superscript within a row are significantly different as determined by ANOVA with least squares means test ($P<0.05$).

Table 2-4 Plasma and hepatic lipid levels in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d †

(Mean values with their standard errors (SEM) for six to ten rats per group)

	BBn (*n* 6)		BBd (*n* 6)		BBdp (*n* 10)	
	Mean	SEM	Mean	SEM	Mean	SEM
Plasma (mmol/L)						
Triglyceride	0.81^{a}	0.09	1.37^{b}	0.23	1.04^{ab}	0.14
Total cholesterol	1.65^{a}	0.08	2.24^{b}	0.12	2.31^{b}	0.05
HDL-cholesterol	1.21^{a}	0.11	1.22^{a}	0.11	1.29^{a}	0.06
Liver (μmol/g)						
Triglyceride	7.79^{a}	1.06	9.37^{a}	1.36	8.69^{a}	0.65
Total cholesterol	6.95^{a}	0.21	6.41^{b}	0.14	5.49^{c}	0.13
Free cholesterol	6.04^{a}	0.13	5.39^{b}	0.14	4.58^{c}	0.11

† BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. BBd and age-matched BBn rats were killed at the time of diabetes onset. The average onset age of diabetes was 95.3 ± 7.4 d with the earliest at 61 d and the latest at 113 d. BBdp rats were killed at the age of 120 d when the experiment was terminated.

Mean values that do not share a common superscript within a row are significantly different as determined by 2-way ANOVA with least squares means test ($P<0.05$).

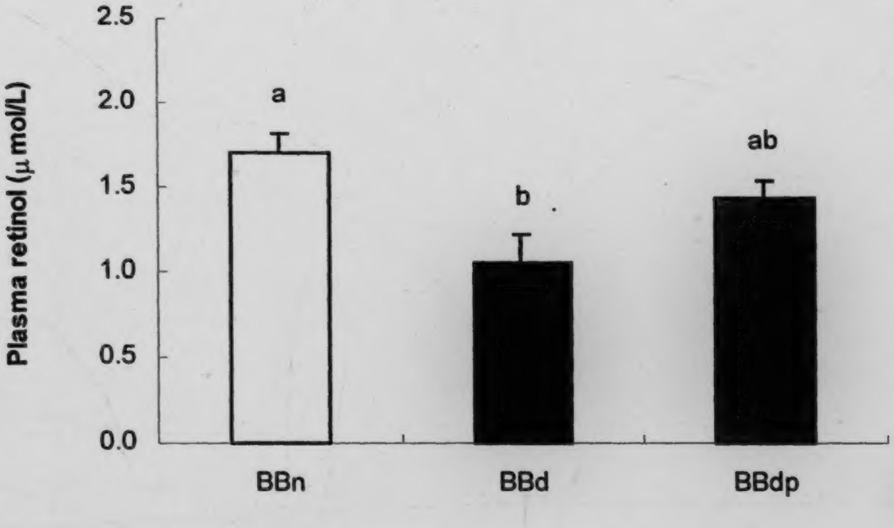

Figure 2-5 Plasma retinol levels in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d.

BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to ten rats with their standard errors shown by vertical bars. Bars that do not share a common letter are significantly different (*P*<0.05).

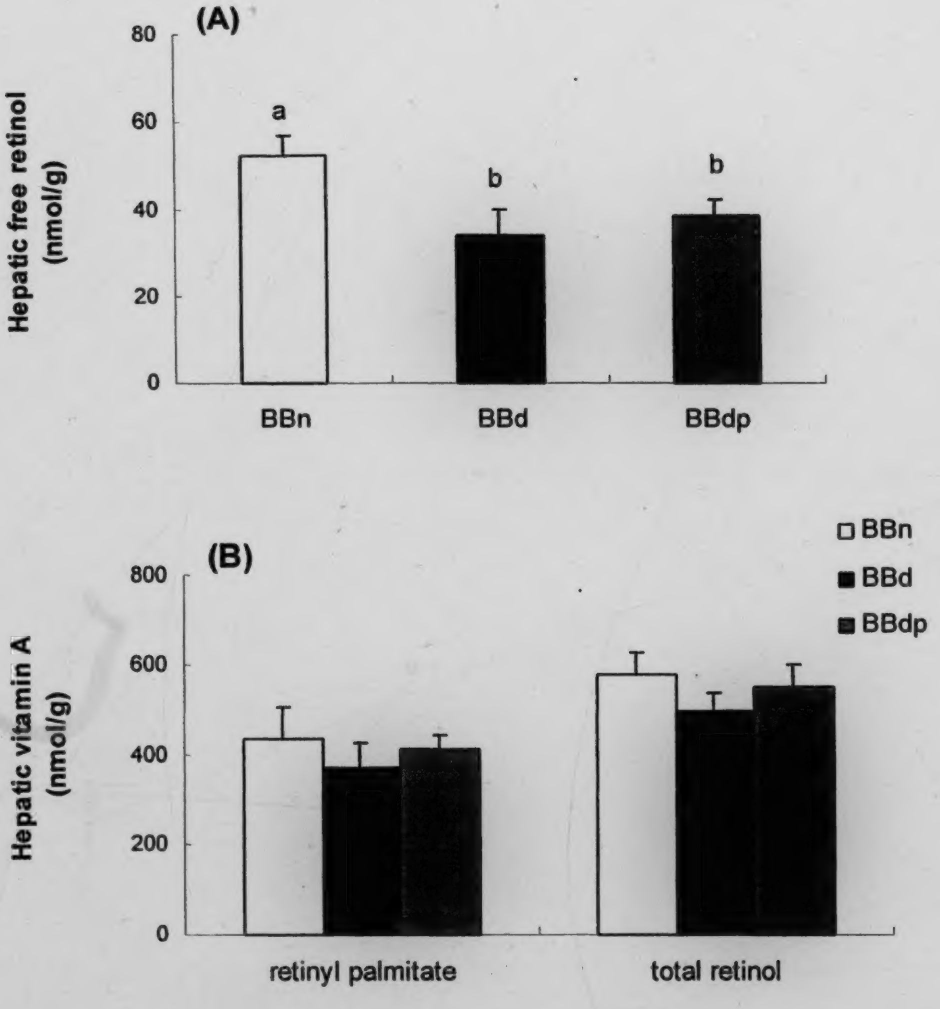

Figure 2-6 Hepatic free retinol (A) and hepatic retinyl palmitate and total retinol (B) levels in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d.

BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to ten rats with their standard errors shown by vertical bars. Bars that do not share a common letter within each panel are significantly different ($P<0.05$). No significant differences were detected in hepatic retinyl palmitate levels and total retinol concentrations among BBn, BBd and BBdp rats.

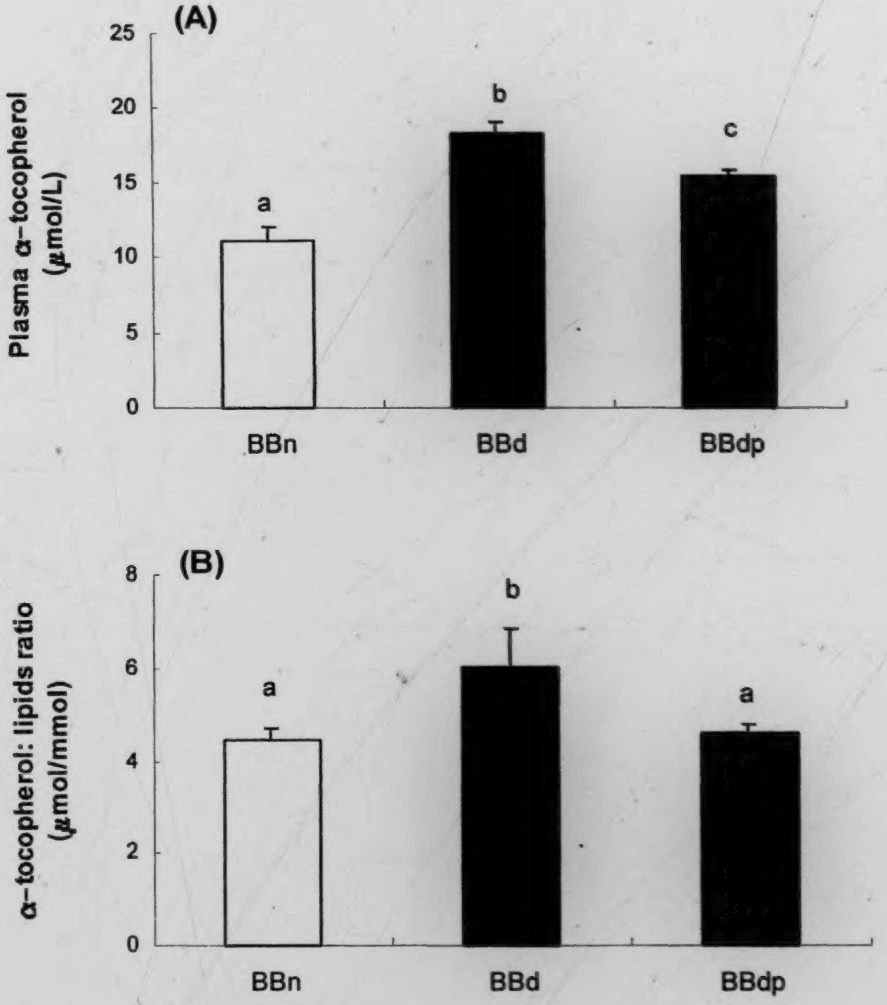

Figure 2-7 Plasma α-tocopherol levels (A) and α-tocopherol: lipids ratio (B) in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d.

BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Plasma lipids are the sum of plasma triglyceride and total cholesterol. Values are means of six to ten rats with their standard errors shown by vertical bars. Bars that do not share a common letter within each panel are significantly different (*P*<0.05).

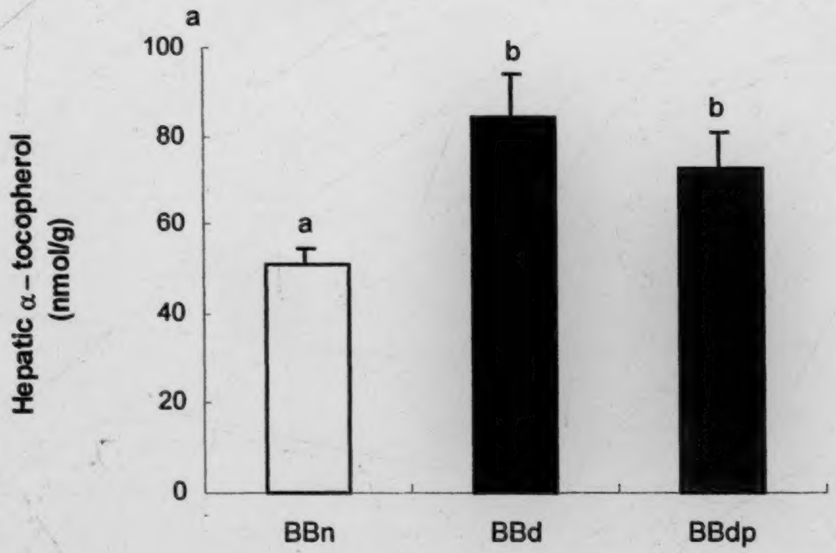

Figure 2-8 Hepatic α-tocopherol levels in BBd rats at the onset of diabetes, the age-matched BBn rats, and BBdp rats aged 120 d.

BBd rats, diabetic BB rats. BBdp rats, diabetes-prone BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of six to ten rats with their standard errors shown by vertical bars. Bars that do not share a common letter are significantly different (*P*<0.05).

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Chapter 3

The hepatic retinyl ester hydrolase activity is depressed at the onset of diabetes

3.1 Introduction

Vitamin A is stored primarily in hepatic stellate cells in the form of retinyl esters, of which about 80% is retinyl palmitate (Batres and Olson 1987). The retinyl esters need to be hydrolyzed to liberate retinol, which is then transported to the circulation by its carrier protein RBP as a retinol-RBP complex. The previous chapter reported that in parallel with a decreased plasma retinol levels, the hepatic free retinol levels were decreased, whereas the ratios of retinyl palmitate to free retinol in the liver were increased in diabetic BB rats. However no significant difference was observed in the hepatic concentration of retinyl palmitate, the major form of vitamin A stores in the liver, between the diabetic rats and normal controls. These results, together with the data from other animal studies (Tuitoek et al. 1996b;Lu et al. 2000), suggest that the decreased plasma retinol level in type 1 diabetes is not likely related to the decreased intestinal or hepatic uptake of retinol, but associated with altered vitamin A mobilization from the hepatic stores.

Two major processes are thought to be involved in the mobilization of retinol from its hepatic storage pools (Blaner and Olson 1994). Firstly, retinyl esters stored in the stellate cells must undergo hydrolysis to release free retinol. Secondly, the liberated retinol binds to RBP and is secreted into the circulation as a retinol-RBP complex. In the blood, RBP-retinol circulates as a 1:1 molar complex with another protein, TTR. The retinol bound to RBP has been considered to be the physiologically important form of the vitamin transported in plasma and utilized by the target tissues (Blomhoff et al. 1990). Reduced plasma concentrations of retinol and RBP in the presence of a hyperglycemic state have been found in animal experiments (Lu et al. 2000;Tuitoek et al. 1996a) and clinical studies (Basu et al. 1989;Krill et al. 1997). Concurrently, it has been reported that the hepatic expression of RBP mRNA is markedly decreased at the onset of diabetes in BB rats (Lu et al. 2000). Those results point to the possibility that vitamin A deficiency in the presence of diabetes may be caused, at least in part, by a decreased transport of the vitamin from its hepatic stores due to limited availability of RBP.

However, it is possible that vitamin A homeostasis in the presence of diabetes is affected not only by an altered retinol carrier protein, but also by the hydrolysis of retinyl ester. As a key step in vitamin A metabolism, retinyl ester hydrolysis is involved in the digestion and intestinal absorption of dietary vitamin A as well as hepatic uptake, storage and mobilization of vitamin A. Decreased retinyl ester hydrolysis in the intestine could lower the amount of free retinol to be taken-up by enterocytes, whereas in the liver the decreased retinyl ester hydrolysis could limit the amount of free retinol to be liberated from its hepatic retinyl ester storage pools and thereby causing less retinol to be secreted into the circulation. So far, the relationship between diabetes and retinyl ester hydrolysis has not been adequately studied.

Research by Tuitoek et al. (1994) examined intestinal absorption of retinol *in vitro* in STZ-induced diabetic rats. No significant differences were found in the rate of intestinal uptake of retinol between the diabetic and the control animals when the concentration of retinol was varied from 1 to 24 μmol/L. However, the main form of dietary vitamin A is retinyl ester, which needs to undergo hydrolysis in order to release free retinol before the retinol is taken up by enterocytes through a saturable, carrier-mediated process (Hollander and Muralidhara 1977;Hollander 1981). With the possible activity change of the enzymes responsible for retinyl ester hydrolysis in intestine, differences might exist in intestinal retinyl ester uptake, whereas intestinal retinol absorption per se may remain unaffected.

The main purposes of this study were therefore to examine: 1) the change of retinyl ester hydrolases in the liver as well as the intestine in the presence of diabetes; 2) the intestinal uptake of retinyl ester *in vitro* in diabetic BB rats.

3.2 Materials and methods

Materials

The chemical reagents were from Sigma-Aldrich Canada Limited (Oakville, Ontario, Canada) and Fisher Scientific Canada (Nepean, Ontario, Canada) unless otherwise stated.

Animals and diet

The animal protocol was reviewed and approved by the same committee described in chapter 2. The source of animals was also described earlier (chapter 2).

Thirty weanling male BBdp rats and thirty age-matched male BBn rats were housed individually in stainless steel, wire-bottom cages in a temperature and humidity controlled animal room with a 12-h light/dark cycle. The rats were given free access to water and an open-formula, nonpurified NIH-07 basal diet in meal form (Ziegler Brothers, Gardner, Pennsylvania, USA). The complete composition of NIH-07 diet was described in Chapter 2. Throughout the experiment, food intake was recorded every other day and weight was recorded weekly.

Beginning at age 50~52 d, a plastic container was placed underneath a spout on the bottom of each cage to collect urine. Urine samples from BBdp rats were tested 3 times/wk for glucose using Chemstrip uG (Boehringer Mannheim, Laval, Quebec, Canada) until the onset of diabetes, or until the rats were 120 d old at which time the experiment was ended. When the glucose levels in urine greater than 56 mmol/L were detected in BBdp rats, blood glucose from the tail vein was measured in the fed state using a glucometer (Ames Miles, Toronto, Ontario, Canada). The onset of diabetes was defined as the time when blood glucose greater than 13.8 mmol/L was detected in fed state (Hosszufalusi et al. 1993). BBdp rats at the onset of diabetes were described as BBd rats.

Each newly diagnosed BBd rat, along with one age-matched BBn control rat was terminated within 1 to 2 d of diabetes onset. The BBd rats and their control animals were terminated, by turns, with or without an over-night fast. Samples from fasting animals were used for the analysis of intestinal and hepatic REH activities and other biochemical indices, while fresh intestine from the fed rats was used for retinyl palmitate uptake study *in vitro*. At age 120 d, all remaining BBdp rats that did not develop diabetes and control animals were terminated following an over-night fast.

Blood and tissue sample collection

Before termination, over-night fasted rats were first anesthetized with 5% (v/v) halothane (Halocarbon Laboratories, River Edge, New Jersey, USA). A blood sample was removed via cardiac puncture and collected with a heparinized tube before rats were terminated via cervical dislocation. Plasma was later separated by centrifugation and stored at –70°C.

After the rats were terminated, livers were quickly removed, rinsed with cold saline (4°C), blotted, and weighed. A portion of the liver sample was left on ice and used for microsomal preparation immediately after the tissue collection on the same day (see later in 'Non-specific carboxylesterase activity' for details). The hepatic microsomes would be used for the measurement of non-specific carboxylesterase activity later. Proximal and distal intestine samples (15 cm length each) were cut off as jejunum and ileum and flushed with ice-cold saline. The retina of the eye was isolated by the method of Uehara et al. (1989). Briefly, the corneas were pierced and dissected with the tip of a razor blade. Slight pressure was then applied to the eye cup using forceps to enable the lens and vitreous body to be extracted. When the lens was extruded, the retina came out with the lens and was washed with several drops of cold saline after it was detached from the lens. All the samples were quickly put into separate plastic vials and frozen immediately in liquid nitrogen before being stored at –70°C for subsequent analysis.

For the study of *in vitro* uptake of retinyl ester, rats without an over-night fast were killed by an intraperitoneal injection of euthanyl (pentobarbital sodium). Intestinal segments (jejunum and ileum) were quickly removed, flushed with ice-cold saline buffer, and used immediately to test the rate of retinyl ester uptake *in vitro* (see later in 'Intestinal uptake of retinyl palmitate' for the detailed method).

Vitamin A and E determination

Plasma and hepatic free retinol, α-tocopherol, and hepatic retinyl palmitate were extracted without saponification and determined by reverse-phase HPLC according to the methods of Tuitoek et al. (1996b), Wang et al. (1998) and Vliet (1991) as described in chapter 2.

Briefly, liver samples were first homogenized with PBS buffer (0.05 mol/L, pH 7.4). Retinoids and α-tocopherol were extracted and injected into a reverse phase Varian 5000 HPLC system (Varian Canada Ltd., Mississauga, Ontario, Canada). A 5 μm Supelcosil LC-18 column (0.46 × 15 cm; Supelco, Ontario, Canada) with a C_{18} guard column was used to separate the vitamins. A Waters 486 tunable absorbance detector (Waters Chromatography Division, Millipore, Milford, Masschusetts, USA) was set at 325 nm for retinol and retinyl esters. A Shimadza RF-535 fluorescence HPLC monitor (Shimadza Corporation, Japan) provided excitation at 295 nm and emission at 330 nm was used to identify α-tocopherol.

The mobile phase used for the plasma samples was methanol-water (95: 5, v/v) at a flow rate of 1.5 ml/min. The mobile phase used for the liver samples consisted of acetonitrile-tetrahydrofuran-water (solvent A: 50:20:30; solvent B: 50:44:6, v/v/v, with 1% (w/v) ammonium acetate and 0.35% (v/v) acetic acid in water) at a flow rate of 1 ml/min. The gradient procedure for the latter mobile phase was described in chapter 2.

Glucose and lipids analysis

Plasma glucose level was measured by the glucose oxidase method using Sigma kit (Procedure No. 315). Plasma triglyceride and total cholesterol levels were measured using enzymatic kits from Sigma (Procedure No. 336 and 401). Plasma HDL-cholesterol was determined using the same kit for total cholesterol measurement in the supernatant of plasma sample after VLDL and LDL were precipitated using HDL cholesterol reagent from Sigma (Catalog No. 352-4).

Liver triglyceride, total cholesterol and cholesterol ester were measured by the enzymatic methods adapted from Carr et al. (1993), Omodeo-Salè et al. (1984) and Radin (1981), as described in chapter 2.

All assays were performed in 96-well microtiter plates and the plates were read on a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA).

Retinyl ester hydrolase (REH) activities

- CHAPS-stimulated REH

The bile salt analog CHAPS-stimulated REH activity was measured in the liver and the intestine according to the method of Cooper et al (1987;1990).

Assay conditions: the conditions for measuring CHAPS-stimulated REH activity were adapted from Cooper et al. (1987) and Mercier et al. (1990). The optimal assay conditions were determined in our laboratory prior to the enzyme analysis using hepatic tissues from BBn rats. From the results (Figure 3-4), the optimal pH appeared to be 7.5. CHAPS (200 mmol/L) and the substrate retinyl palmitate (1.5 mmol/L) concentrations were chosen to give simultaneously a maximal enzyme activity and a minimized variation coefficient. Protein concentration (300 μg / 0.2 mL) and incubation time (90 min) were in the linear part of the curve. Under these conditions, kinetic parameters for REH activity were determined, which showed an apparent *K*m of 0.54 mmol/L and a maximal velocity of 10.8 nmol of retinol formed per hour (Figure 3-5).

Tissue preparation: tissues (liver, jejunum or ileum) were weighed (0.3 to 0.5 g) and homogenized with 3 volumes (w/v) of ice-cold Tris-maleate (2-amino-2-hydroxymethylpropane-1,3-diolmaleate) buffer (50 mmol/L, pH 7.2) with a motor-driven mortar and pestle (Tri-R Instruments, Rockville Center, New York, USA) in an ice-water bath for 30 to 40 seconds. These homogenates can be frozen in liquid nitrogen and stored at –20°C for at least 1 year without loss of activity (Cooper 1990).

Protein analysis: total protein concentrations in tissue homogenates were quantified by using the Bicinchoninic Acid Protein Assay kit (Pierce Co., Rockford, Illinois, USA). Bovine serum albumin was used as the protein standard.

Substrate preparation: retinyl palmitate (15 mmol/L) was dissolved in 2% (v/v) Triton X-100 (in 100 mmol/L Tris-maleate buffer, pH 7.5) by stirring vigorously with a small stir bar. The substrate mixture was used on the same day of its preparation and discarded afterwards.

Assay procedure: an aliquot of tissue homogenates (about 300 μg of protein) was incubated in 100 mmol/L Tris-maleate buffer (pH 7.5) in a shaking water bath at 37°C for 90 min. The incubation mixture also contained 200 mmol/L CHAPS in 100 mmol/L Tris-maleate and 1.5 mmol/L retinyl palmitate substrate, with a total final volume of 0.2 ml. The reaction was stopped by the addition of 0.4 ml of ethanol after the incubation period. Retinol in the incubation was then extracted and measured following the same HPLC procedure for liver vitamin A determination (see above in 'Vitamin A and E determination').

Activity calculation: REH activity was calculated from the amount of retinol produced during the assay. Control incubations without homogenate allowed correction for non-enzymatic retinol formation. Endogenous retinol from the homogenates was verified to represent less than 0.1% of the retinol measured. The enzymatic activity of REH was expressed as the nanomoles of retinol liberated per milligram of protein per h.

- Bile salt-independent REH

Bile salt-independent REH activities in the liver, the intestine and the retina were conducted according to Tsin et al. (2000;1992).

Tissue preparation: the microsomal fraction was prepared from the tissue by differential centrifugation. Tissue samples of liver and intestine were homogenized in 4 volumes (w/v) of ice-cold buffer A (0.25 mol/L sucrose, 25 mmol/L Tris-acetate, 2 mmol/L dithiothreitol (DTT), 2 mmol/L Ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 7.0) using a Polytron homogenizer (Brinkmann Instruments; Wesbury, New York, USA; setting 6 for 20 seconds). Homogenates were first centrifuged at 27,000 g for 20 min at 4°C (Sorvall RC-5B superspeed centrifuge; Dupont Instrument, Wilmington, Delaware, USA). The resulting supernatant was carefully decanted and centrifuged at 125,000 g for 90 min at 4°C (Beckman Optima LE-80K ultracentrifuge; Beckman Coulter, Inc., Fullerton, California, USA). The pellet was resuspended in ice-cold 10 mmol/L Tris-acetate buffer B (pH 7.5, containing 2 mmol/L DTT and 2 mmol/L EDTA) and homogenized in a glass Potter-Elvejhem homogenizer. Retinas were homogenized in a glass homogenizer in 3 ml of ice-cold buffer A and centrifuged at 125,000 g for 90 min at 4°C. Pellets were resuspended in ice-cold buffer B. Protein content in the microsomal material was then determined.

Assay procedure: the reaction mixture included 2 nmol of [^{3}H] 11-cis-retinyl palmitate delivered in 10 μl of ethanol, 1-5 μg of microsomal protein and 50 mmol/L Tris-acetate buffer (pH 8). The final volume of assay mixture was 200 μl. After a 30-min incubation at 37°C, the reaction was stopped by the addition of 3 ml of methanol-chloroform-heptane (1.41:1.25:1, v/v/v), followed by 1 ml of 50 mmol/L potassium carbonate buffer (pH 10). After the reaction mixture was mixed and centrifuged at 1000 rpm for 15 min at 4°C, 1 ml of the aqueous upper phase ([^{3}H] palmitic acid) was removed, mixed with 10 ml Econo-1 scintillation cocktail (Fisher Scientific, Fair Lawn, New Jersey, USA), and analyzed for [^{3}H] using a liquid scintillation counter.

Activity calculation: Bile salt-independent REH activity was calculated as picomoles of palmitic acid liberated per milligram of protein per min. Background correction was carried out in each analysis by incubating the substrate with heat-denatured microsomal protein.

- Non-specific carboxylesterase activity

Non-specific carboxylesterase activities in liver were measured by the method adapted from Chanda et al. (1997) and Zhang & Fariss (2002).

Assay conditions: conditions for the assay were adapted from Chanda et al. (1997) and determined in our laboratory prior to the enzyme analysis. Tissue concentration and incubation time were chosen to produce linear rates of substrate hydrolysis. The optimum substrate concentration in the incubation was obtained by incubating the tissue with different concentrations of substrate (Figure 3-7 A).

Tissue preparation: hepatic microsomes were promptly prepared after the livers were collected from rats according to the method of Ong et al. (1987;1988). The liver tissue was first homogenized in a ice-water bath with motor-driven teflon pestle in 3 volumes (w/v) of 0.25

mol/L sucrose buffered with 0.01 mol/L potassium phosphate (pH 7.4). The homogenates were centrifuged at 4°C for 10 min at 750 g. The supernatants were then centrifuged at 3000 g for 10 min in a JA-20 rotor of a Beckman centrifuge (Beckman J2-21 centrifuge, Beckman Instruments, Inc., Spinco Division, Palo Alto, California, USA). After floating fat was removed, the resulting supernatants were again centrifuged in a Beckman L8-70M Ultracentrifuge using a Ti70.1 rotor at 113,000 g for 60 min. The recovered pellet was resuspended in 0.2 mol/L potassium phosphate buffer, pH 7.4, with 1 mmol/L dithiothreitol to a final concentration of 15-25 mg protein/ml and quickly frozen before storage at -70°C.

The protein content of microsomal preparations were determined by using the Bicinchoninic Acid Protein Assay kit (Pierce Co., Rockford, Illinois, USA). Bovine serum albumin was used as the protein standard.

Assay procedure: the non-specific carboxylesterase activity was determined at 26°C by measuring the formation of *p*-nitrophenol from *p*-nitrophenyl acetate. The assay was performed in 96-well, flat-bottom polystyrene microtiter plates using a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA). Hepatic microsomes were first diluted with 0.1 mol/L potassium phosphate buffer, pH 7.4. The samples (10μl) were then added to 90 μl of Tris buffer (50 mmol/L, pH 8.0) in the microtiter plate. One hundred microliters of substrate, *p*-nitrophenyl acetate (1.8 mmol/L), was added to each well after a 5 min pre-incubation at room temperature. The increase in absorbance at 405 nm was recorded for 10 min (with a reading every 10 sec) after the substrate was added. Control incubations without tissue samples were run simultaneously to account for the spontaneous hydrolysis of *p*-nitrophenyl acetate.

A standard curve of *p*-nitrophenol was established by adding different amounts of *p*-nitrophenol (1-40 nmol) and measuring the optical density at 405 nm (Figure 3-7 B).

Activity calculation: non-specific carboxylesterase activity was calculated from the amounts of *p*-nitrophenol produced during the assay and expressed as nanomoles of *p*-nitrophenol formed per microgram of protein per min.

Intestinal uptake of retinyl palmitate

The intestinal uptake of retinyl ester was determined following the procedure described by Tuitoek et al. (1994). Immediately after termination of BBd rats and corresponding control (BBn) rats, a 15-cm length of proximall jejunum and 15-cm length of distal ileum were rapidly removed from the rats and rinsed gently with cold saline. The intestinal segment was opened along its mesenteric border and the mucosal surface was flushed with cold saline to remove visible mucus and debris. Circular disks of the intestine were cut from each segment, mounted as flat sheets in the incubation chambers, and pre-incubated in oxygenated Krebs buffer at 37°C for 15 min.

Following the pre-incubation, the chambers were transferred to beakers containing [carboxyl-^{14}C] inulin (NEN Life Science Products, Inc., Boston, Massachusetts, USA), and [15-^{3}H] retinyl palmitate (American Radiolabeled Chemicals, Inc., St Louis, Missouri, USA). The concentrations of retinyl palmitate ranged from 32 nmol/L to 256 nmol/L. After incubation for 6 min at 37°C, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for approximately 5 seconds. The exposed mucosal tissue was cut out of the chamber with a circular steel punch and placed on glass slides. The tissue was then dried overnight at 55°C and the dry weight of each sample was determined afterwards. The dried sample was saponified with 0.1 mol/L sodium hydroxide. Scintillation fluid was then added, and the radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes. The effect of the unstirred water layer was minimized by stirring solutions at 600 rpm with a circular magnetic bar. The presence of any adherent mucosal fluid was assessed by the presence of the non-absorbable marker, [carboxyl-^{14}C] inulin, added in the incubation buffer.

Statistical analysis

Results were expressed as mean values with the standard errors. All statistical analyses were conducted using the SAS system (SAS Institute, Cary, North Carolina, USA). Student's t test was used to determine the difference between diabetic BB rats and non-diabetes control rats at the onset of diabetes. A two-tailed *P*-value of ≤ 0.05 was considered significant.

3.3 Results

General characteristics, lipids, vitamin A and E

By the end of the experiment, when rats reached the age of 120 d, thirteen out of the thirty BBdp rats had developed diabetes. The incidence of diabetes in these BBdp rats was 43.3%. The earliest and latest ages of diabetes occurrence were 61 and 105 d, respectively, with an average onset age at 81 d. All of the BBd rats and their corresponding non-diabetes controls were killed within 1 or 2 d of diabetic onset.

Results from Table 3-1 showed that the general characteristics including average daily food intake and final body weight gain of BBd rats did not differ significantly from that of their age-matched BBn controls. Weights of liver tissue between the two groups of rats were also quite similar at the onset of diabetes. As expected, urine excretion and plasma glucose levels were significantly higher in BBd rats compared with their age-matched non-diabetic controls.

Plasma total and HDL-cholesterol concentrations were significantly higher in the presence of diabetes in BB rats. No difference was found in plasma or hepatic triglyceride levels between BBd and BBn rats. However, the hepatic total cholesterol levels were significantly lower in BBd rats compared with that of age-matched BBn rats (Table 3-2). No difference was observed in hepatic free cholesterol level between these two groups of rats.

Compared with the age-matched BBn controls, BBd rats had markedly decreased plasma as well as hepatic free retinol levels (Figure 3-1 and Figure 3-2, A). However, the hepatic retinyl palmitate concentrations did not differ significantly between diabetic BB rats and their counterparts (Figure 3-2, B). The molar ratio of retinyl palmitate to retinol in the liver was higher in BBd rats than in BBn rats (19.97±3.02 and 11.46±0.91, respectively, $p<0.05$).

Unlike the plasma and hepatic retinol levels, vitamin E concentrations in either the plasma or the liver were significantly elevated in diabetic BB rats (Figure 3-3).

Retinyl ester hydrolase activity

Several retinyl ester hydrolase activities, including CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase, were measured in the intestines and the livers of BB rats.

Results showed that the hepatic activities of CHAPS-stimulated REH were significantly decreased in diabetic BB rats compared with that of the BBn controls (Figure 3-6, A). The 95% CI for hepatic activity of CHAPS-stimulated REH was 2.0 to 4.8 in diabetic BB rat, and 4.2 to 6.0 in non-diabetic control rats. However, the changes in CHAPS-stimulated REH activity were only found in the liver, and were not present in the jejunum and ileum of the animals (Figure 3-6, A).

Bile salt-independent REH activity was determined in the liver, jejunum, ileum and retina (Figure 3-6, B). Results showed that the hepatic bile salt-independent REH activities were significantly reduced in diabetic BB rats compared to those of the normal BBn controls. The 95% confidence interval (CI) for hepatic bile salt-independent REH activity was 467 to 591 in BBd rats and 583 to 681 in BBn controls. Similar to the results of CHAPS-stimulated REH activity, no change in bile salt-independent REH activity was found in the jejunum and the ileum in the presence of diabetes. The bile salt-independent REH activities in the retina did not differ between diabetic BB rats and their control counterparts. Limited by the amount of sample, the CHAPS-stimulated REH activity was not measured in the retina in the present study.

Consistent with the change of CHAPS-stimulated REH and bile salt-independent REH activities, results showed that non-specific carboxylesterase activities in the liver were decreased in BB rats in the presence of diabetes (Figure 3-8).

Intestinal uptake of retinyl palmitate *in vitro*

An *in vitro* study comparing the intestinal uptake of retinyl palmitate between diabetic BB rats and age-matched non-diabetic BBn control rats was carried out in this study. Freshly removed jejunal and ileal portions of the intestine from diabetic BB rats and the control animals were incubated with labeled retinyl palmitate at different concentrations, which were within the physiological range according to the published data (< 300 nmol/L) (Hollander and Muralidhara 1977).

Results demonstrated that, at concentrations of retinyl palmitate between 32 to 256 nmol/L in the incubation, there was a linear relationship between increasing concentrations of retinyl palmitate, and the rate of retinyl palmitate uptake into both the jejunum and ileum (Figure 3-9). However, no difference was observed between BBd and BBn rats in the rate of retinyl palmitate uptake at these concentrations. The slope of the line between uptake of retinyl palmitate and the concentration of retinyl palmitate (32 to 256 nmol/L) was similar in diabetic animals and the controls in the jejunum (15.4±1.6 and 20.1±3.3, respectively, $P>0.05$) and the ileum (23.9±4.5 and 20.5±1.8, respectively, $P>0.05$).

3.4 Discussion

One of the purposes of this study was to examine the changes in biochemical status of retinol as well as REH activities in BB rats in the presence of diabetes. Consistent with the results from chapter 2, diabetic rats showed decreased circulatory and hepatic free retinol levels at the onset of diabetes although the hepatic retinyl palmitate concentrations in these rats did not differ compared with normal control animals. The molar ratios of retinyl palmitate to retinol were significantly higher in diabetic BB rats. These results suggest that less retinol is liberated from its ester form in the presence of diabetes and therefore may limit the availability of retinol in the liver.

In the process of vitamin A metabolism, retinyl ester hydrolysis plays an important role in either the intestinal uptake of dietary retinyl ester, or the hepatic uptake of chylomicron remnant-associated retinyl ester, as well as the mobilization of stored retinyl ester from the liver. A number of REH activities have been described in some tissues, such as the intestine, pancreas, liver, kidney, lung and the eye (Harrison and Gad 1989;Gueli et al. 1991;Rigtrup and Ong 1992). However, the physiological roles of individual REH activity in the hydrolysis of retinyl esters are far from clear.

In the intestine, bile salt-stimulated REH (both pancreas-originated and intrinsically located) and pancreatic triglyceride lipase have been reported in previous studies (Harrison 1988;Rigtrup and Ong 1992;van Bennekum et al. 2000). Several biochemically distinct REHs have been detected in the liver. These include bile salt-dependent REH, CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase (Prystowsky et al. 1981;Cooper et al. 1987;Gad and Harrison 1991;Harrison et al. 1995;Mentlein and Heymann 1987).

Bile salt-dependent REH activity is suggested to hydrolyze the dietary retinyl esters in the intestine and the chylomicron remnant-associated retinyl esters in the liver (Harrison et al. 1995;Lombardo and Guy 1980). It is postulated that bile salt-dependent REH in the intestine is actually pancreatic CEL, which is secreted by the pancreas into the intestinal lumen (Harrison and Gad 1989). Hepatic bile salt-dependent REH is found to be very similar to bile salt-dependent CEL of pancreas origin (Harrison 1988). Newer studies, however, challenge that hypothesis by showing that CEL deficiency has no effect on either dietary retinyl ester hydrolysis in the intestine, or hepatic uptake of chylomicron remnant-derived retinyl ester *in vivo* in CEL gene knockout mice (Weng et al. 1999;van Bennekum et al. 1999). In these CEL gene knockout mice, the uptake of intestinal retinyl esters was normal and uptake of cholesterol ester was about 50% compared with wild-type animals (Weng et al. 1999). Serum clearance of intravenously injected [^{3}H]-retinyl ester from chylomicron did not differ between gene knockout mice and wild-type animals. Study also failed to show any difference in total retinoid and free retinol levels in the liver, or retinol and RBP levels in the plasma, between CEL gene knockout mice and wild-type animals (van Bennekum et al. 1999). These data suggest that another bile salt-dependent REH, distinct from CEL, is responsible for the hydrolysis of retinyl esters in the gut or the liver of the mouse.

Stimulated by a bile salt analog, CHAPS, an REH activity has been reported in pigs (Cooper and Olson 1986;Cooper and Olson 1988), rats (Cooper et al. 1987) and humans (Tsin et al. 1986). One-third of this enzymatic activity has been found in the supernatant fraction of liver homogenates, and the remainder is in the membrane fractions (Cooper and Olson 1986;Cooper et al. 1987). The physiological roles and characteristics of CHAPS-stimulated REH are not yet fully understood. However, the CHAPS-stimulated REH activity appears to be distinct from bile salt-dependent CEL activity based on sequence analysis (Schindler et al. 1998). The CHAPS-stimulated REH activity was determined in this study to represent, at least in part, the bile salt-dependent REH activities in the intestine and the liver.

A neutral, bile salt-independent REH was also measured in the intestine and the liver of BB rats. This bile salt-independent REH has a specific activity for retinyl esters over triglyceride, and it does not catalyze the hydrolysis of cholesterol esters (Napoli et al. 1989;Sun et al. 1997). Studies have found that this enzyme co-localizes with newly delivered chylomicron retinyl esters in the same plasma membrane/endosome fraction in the liver and is evenly distributed in hepatic parenchymal and nonparenchymal cell fractions (Harrison et al. 1995;Matsuura et al. 1997). These results suggest that bile salt-independent REH is involved not only in the initial stage of hepatic hydrolysis of chylomicron retinyl esters, but also in the mobilization of vitamin A from its hepatic stores.

The third enzyme measured in this study is the non-specific carboxylesterase. It has long been reported that non-specific carboxylesterase has retinyl ester hydrolase activity (Mentlein et al. 1987). The non-specific carboxylesterase family includes at least six different isozymes, among which ES-4 has very similar structural, immunological and catalytic features compared to those reported for CHAPS-stimulated REH (Mentlein and Heymann 1987). The amino acid sequences of ES-2 are highly homologous with bile salt-independent REH (Sun et al. 1997). Also, ES-10 may function as a bile salt-independent REH in the liver of rats (Sun et al. 1997). It is still not clear, however, if the corresponding isozyme of non-specific carboxylesterase is identical to CHAPS-stimulated REH or bile salt-independent REH *in vivo*.

This study clearly demonstrates that all three enzymatic activities including CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase were depressed in the liver of diabetic BB rats in parallel with the decreased levels of plasma and hepatic retinol. These findings suggest that the alteration of vitamin A status in the presence of diabetes is not only linked to an inadequate availability of retinol carrier proteins, as reported elsewhere (Lu et al. 2000), but also is associated with the depressed REH activities in the liver in diabetic BB rats. The underlying mechanism(s) for the depressed hepatic REH activity in diabetes is not yet clear. One previous study has reported a 4-fold reduction in retinyl palmitate hydrolase in the liver of rats fed a low protein diet containing 4% casein as the protein source compared with rats fed a control diet containing 20% casein (Tsin et al. 1986). These results suggest that a severe protein deficiency might result in depressed REH activity in the rats. Other evidences suggest that elevated α-tocopherol levels in the liver may contribute to the depressed REH activities. One study has reported that CHAPS-stimulated REH activity or bile salt-dependent REH activity is inhibited by the addition of α-tocopherol *in vitro* (Prystowsky et al. 1981). Another study reported a fifty percent inhibition of hepatic retinyl ester hydrolysis induced by 100 μmol/L α-tocopherol, a concentration close to that in the liver of rats fed moderately low amounts of α-tocopheryl acetate in the diet (Napoli et al. 1984). However, the potential link between depressed REH activity and increased α-tocopherol level needs further investigation.

Results from this study show that the alterations of REH activities were tissue-specific in diabetic BB rats. Despite the depressed REH activities in the liver, the presence of diabetes did not affect CHAPS-stimulated REH activity and bile salt-independent REH activity in either the jejunum or ileum of BB rats. It is noteworthy, however, that in addition to the three REHs measured in the present study, there are other enzymes such as phospholipase B and pancreatic triglyceride lipase that might be involved in the digestion of dietary retinyl esters in the intestine (Rigtrup et al. 1994;van Bennekum et al. 2000).

An *in vitro* intestinal uptake study was therefore conducted using radiolabeled retinyl palmitate to determine the possible REH associated change of dietary retinyl ester uptake in the intestine. Retinyl palmitate was used in the intestinal uptake study because this long-chain fatty acid ester of retinol is the major form of preformed vitamin A in the diet. Dietary retinyl esters must undergo hydrolysis to liberate retinol before the free form of vitamin A can be taken-up by the intestinal enterocyte. This study did not find any significant effect of diabetes on the rate of retinyl palmitate uptake into the intestine when the concentration of retinyl palmitate was within the physiological range as reported elsewhere (Hollander and Muralidhara 1977). A previous study by Tuitoek found that the intestinal uptake of labeled free-retinol was not affected by STZ-induced diabetes in rats (Tuitoek et al. 1994). Overall, these data suggest that the presence of diabetes does not affect vitamin A intestinal absorption including the dietary retinyl ester hydrolysis in intestinal lumen and the free retinol uptake by the intestinal enterocyte. This hypothesis is in agreement with other results from this study, in which diabetic rats had a similar hepatic retinyl palmitate level compared to that of age-matched BBn control animals.

Table 3-1 General characteristics and plasma glucose levels in BBd and BBn rats [†]

(Mean values with their standard errors (SEM) for thirteen rats per group)

	BBn		BBd		Significance, *P**
	Mean	SEM	Mean	SEM	
Food intake (g/d)	17.68	0.75	17.97	0.69	NS
Final body weight (g)	341.6	26.4	335.3	26.9	NS
Liver weight (g)	11.91	1.10	13.63	1.33	NS
24 h urine volume (ml/24 h)	7.36	1.91	76.82	15.46	0.001
Plasma glucose (mmol/L)	8.5	0.31	15.44	0.58	0.013

† BBn rats, non-diabetes-prone BB rats; BBd rats, diabetic BB rats. BBd and age-matched BBn rats were killed at the time of diabetes onset. The average onset age of diabetes was 81.5± 3.9 d with the earliest at 61 d and the latest at 105 d.

* Significant difference between BBd rats and age-matched BBn controls was determined by Student's *t*-test. NS = $P>0.05$.

Table 3-2 Plasma and hepatic lipid levels in BBd and BBn rats †

(Mean values with their standard errors (SEM) for seven rats per group)

	BBn		BBd		Significance, *P**
	Mean	SEM	Mean	SEM	
Plasma (mmol/L)					
Triglyceride	1.31	0.14	1.31	0.24	NS
Total cholesterol	1.48	0.09	2.57	0.38	0.001
HDL-cholesterol	1.19	0.06	1.66	0.18	0.008
Liver (µmol/g)					
Triglyceride	9.32	0.99	10.04	1.06	NS
Total cholesterol	6.16	0.19	5.45	0.28	0.028
Free cholesterol	4.91	0.13	4.69	0.15	NS

† BBn rats, non-diabetes-prone BB rats; BBd rats, diabetic BB rats. BBd and age-matched BBn rats were killed at the time of diabetes onset. The average onset age of diabetes was 81.5± 3.9 d with the earliest at 61 d and the latest at 105 d.

* Significant difference between BBd rats and age-matched normal BBn controls was determined by Student's *t*-test. NS = *P*>0.05.

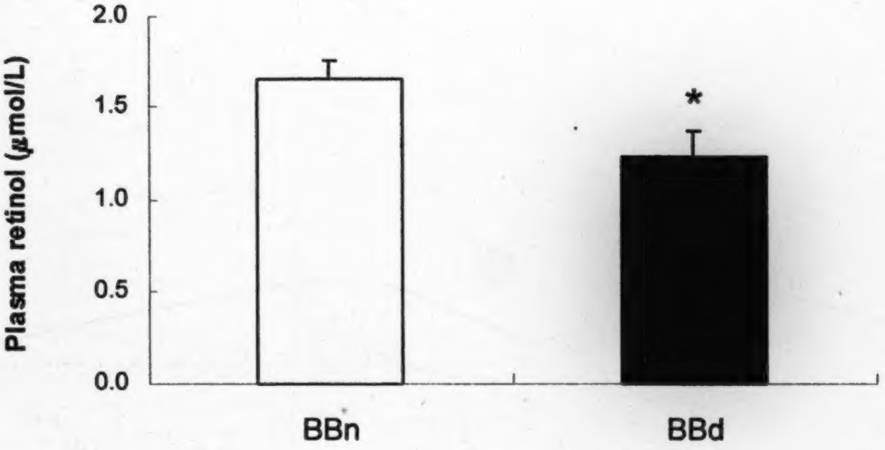

Figure 3-1 Plasma retinol concentrations in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are means for seven rats with their standard errors shown by vertical bars. * indicates a significant difference between BBd and BBn rats ($P<0.05$).

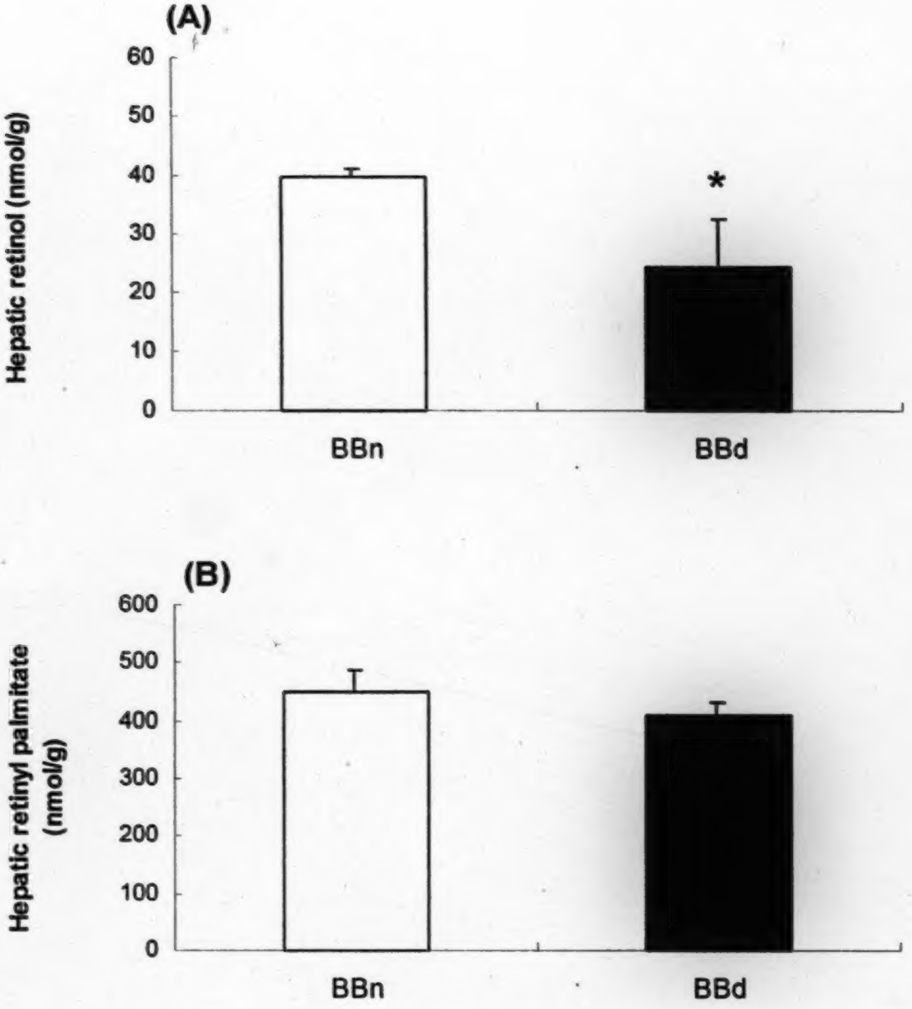

Figure 3-2 Hepatic retinol (A) and retinyl palmitate (B) concentrations in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are means for seven rats with their standard errors shown by vertical bars. * indicates a significant difference between BBd and BBn rats (*P*<0.05).

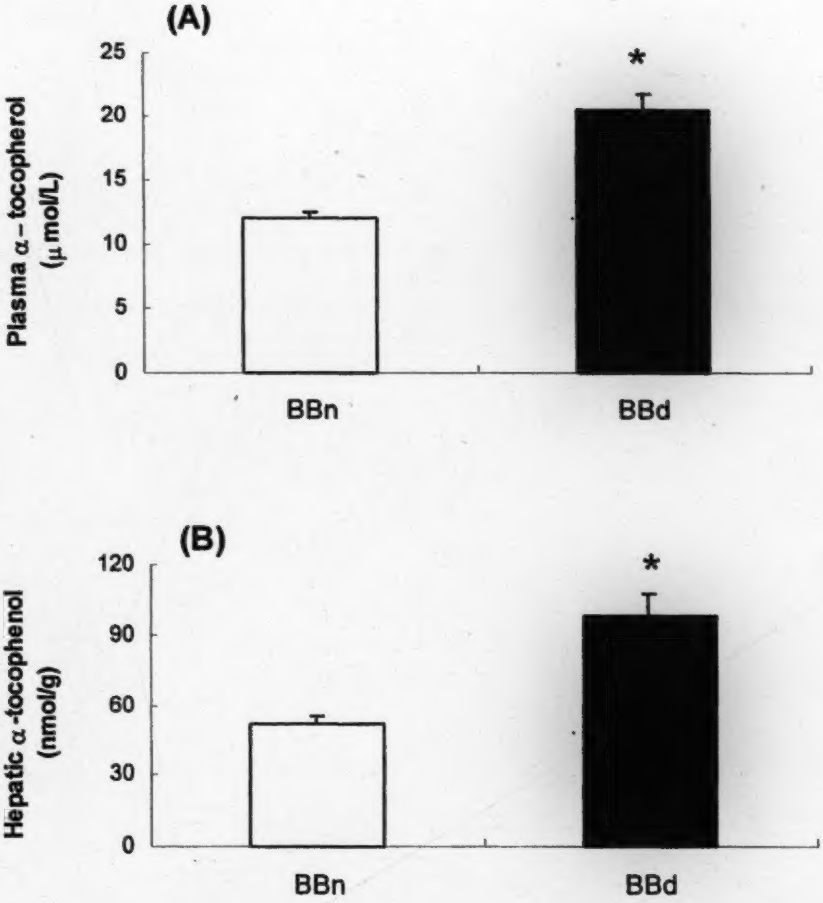

Figure 3-3 Plasma (A) and hepatic (B) α-tocopherol levels in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are means for seven rats with their standard errors shown by vertical bars. * indicates a significant difference between BBd and BBn rats ($P<0.05$).

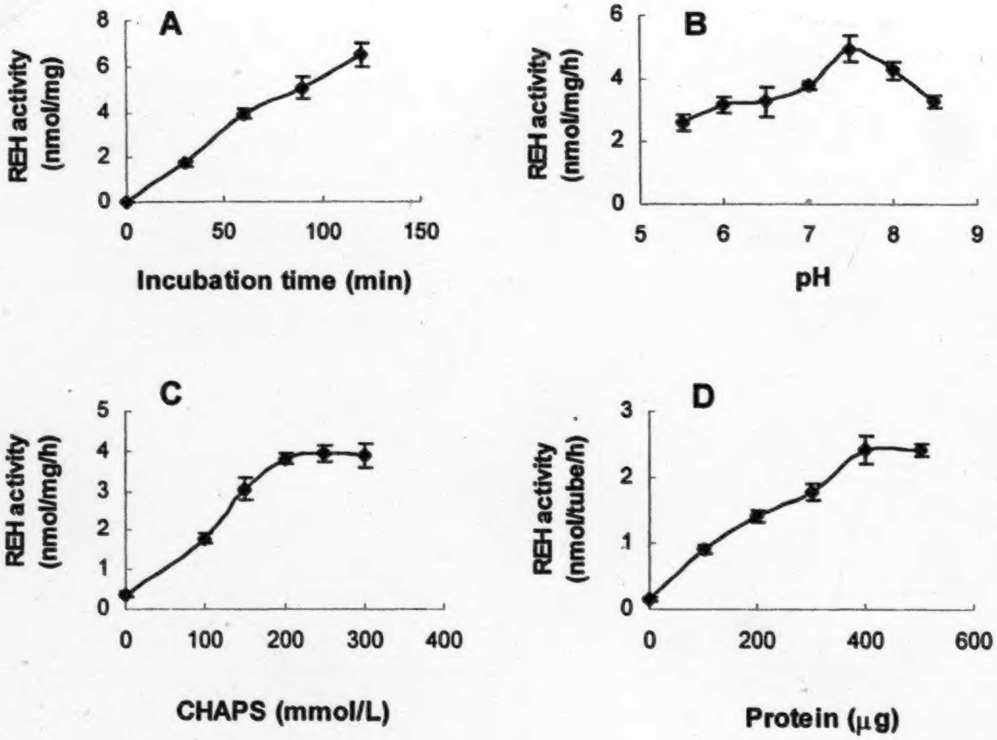

Figure 3-4 Optimal assay conditions for CHAPS-stimulated REH.

Incubations were carried out at 37°C in a final volume of 0.2 ml in Tris-maleate buffer (0.1 mmol/L). (A), effect of incubation time; (B), effect of pH; (C), effect of the CHAPS concentration; (D), effect of protein concentration. Values are means for five or six different incubations with standard errors represented by vertical bars.

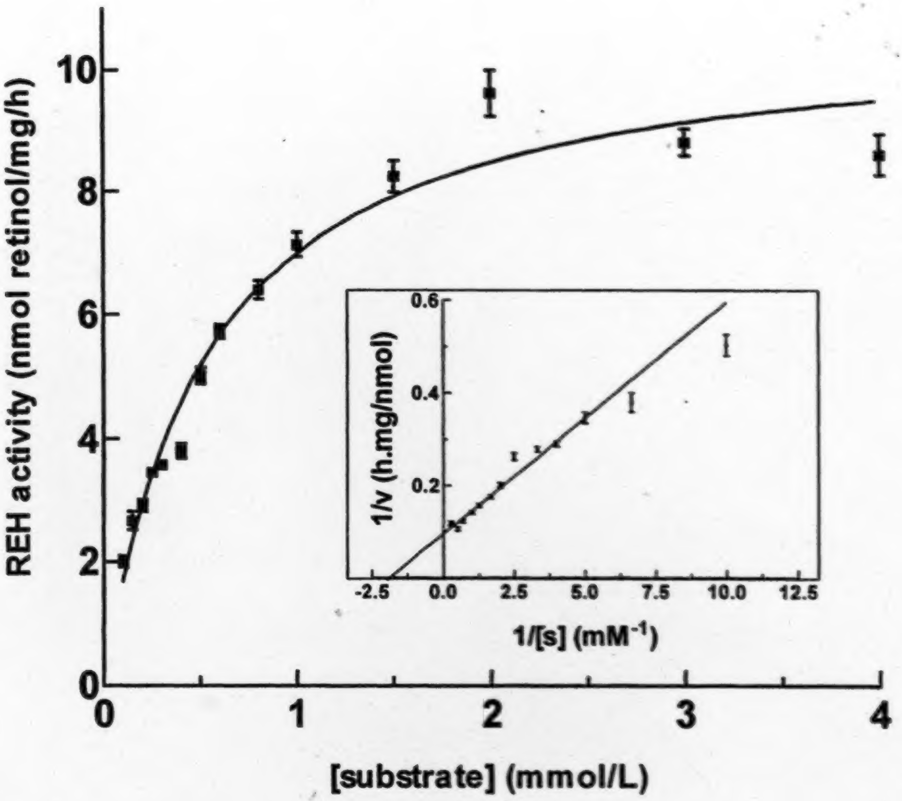

Figure 3-5 Dependence of the CHAPS-stimulated REH activity in the liver on substrate retinyl palmitate concentrations.

Values are means for six different incubations with standard errors represented by vertical bars and expressed as nanomole of retinol liberated per milligram of protein per h. Inset: Lineweaver-Burk plotting of data. [S], substrate concentration; v, initial rate of reaction at a given value of S.

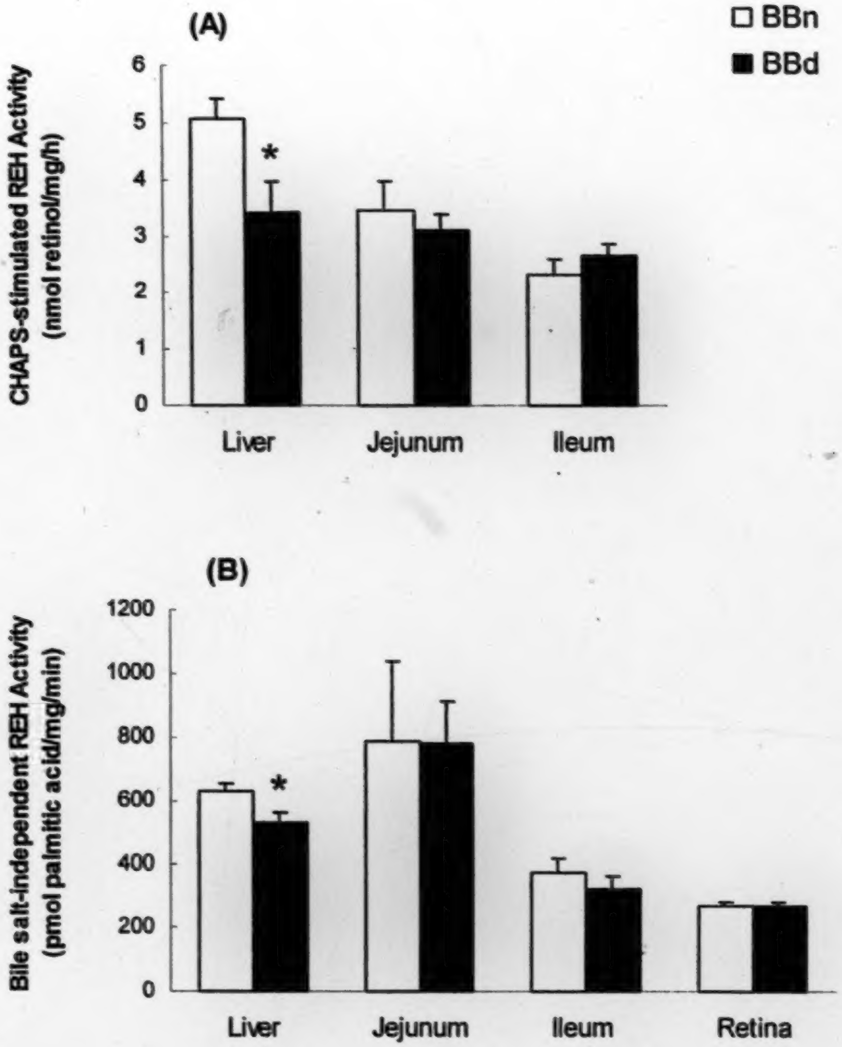

Figure 3-6 Enzymatic activity of CHAPS-stimulated REH (A) and bile salt-independent REH (B) in the tissues in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are means of seven rats with their standard errors shown by vertical bars. * indicates a significant difference between BBd and BBn rats ($P<0.05$).

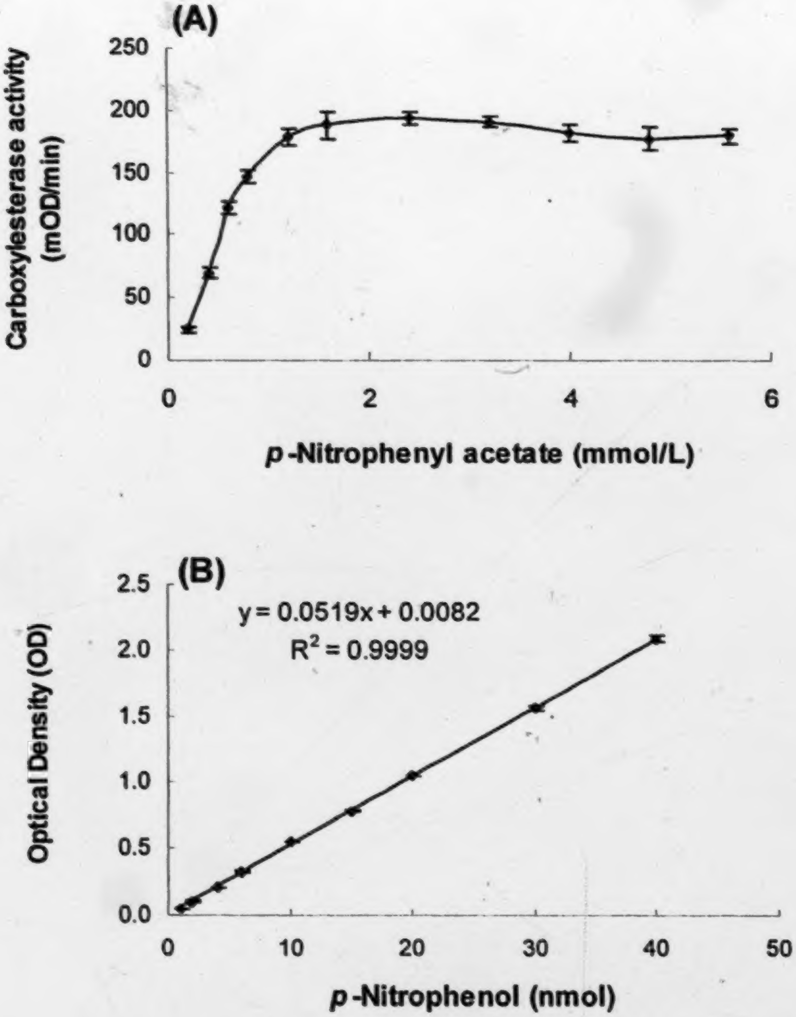

Figure 3-7 Substrate concentration curve (A) and standard curve for *p*-nitrophenol (B) in non-specific carboxylesterase activity assay.

p-nitrophenyl acetate is used as substrate. Values are means of six measurements with their standard errors shown by vertical bars. Non-specific carboxylesterase activity is expressed in panel A as change in optimal density (mOD/min). Change in optimal density of increasing amounts of *p*-nitrophenol was measured at 405 nm. Equation displayed on panel B was used to calculate the non-specific carboxylesterase activity in the experiment.

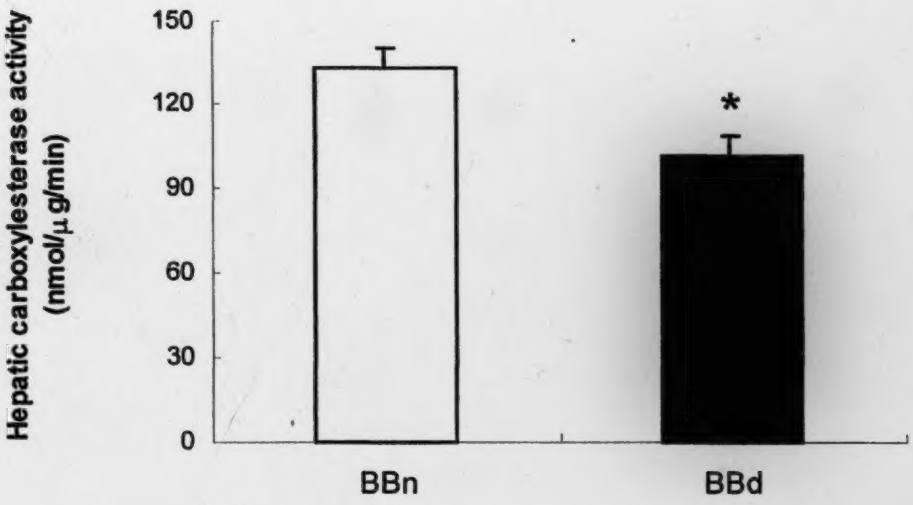

Figure 3-8 Hepatic non-specific carboxylesterase activity in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are expressed as nmol *p*-nitrophenol/μg protein/min. Values are means of seven rats with their standard errors shown by vertical bars. * indicates a significant difference between BBd and BBn rats ($P<0.05$).

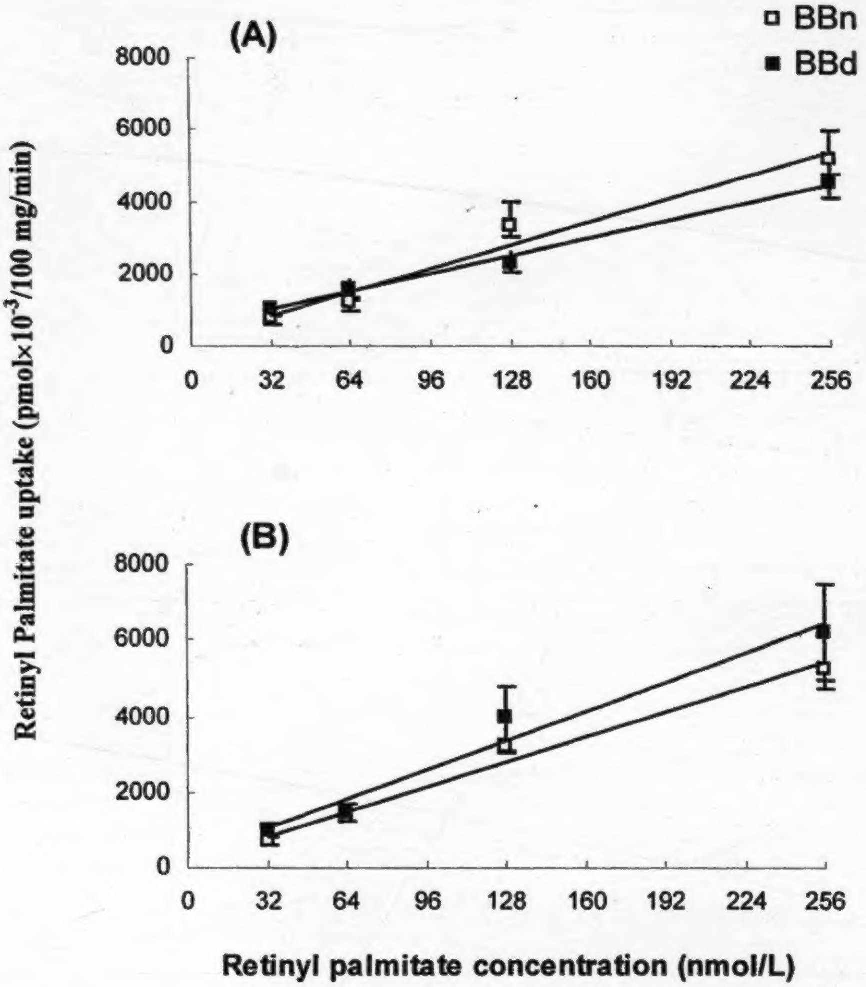

Figure 3-9 The uptake of retinyl palmitate into the jejunum (A) and the ileum (B) at different concentrations of retinyl palmitate *in vitro* in BBd and BBn rats.

BBd rats, diabetic BB rats. BBn rats, non-diabetes-prone BB rats. Values are means for six rats with their standard errors shown by vertical bars. No significant effect of diabetes on intestinal uptake of retinyl palmitate was detected.

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Chapter 4

Modifying effect of dietary vitamin A on vitamin A metabolic status in BB rats

4.1 Introduction

The results in chapter 2 and 3 showed that decreased plasma retinol levels were found not only in diabetic BB rats, but also in diabetic-prone BB rats at the pre-diabetic stage. In parallel with the reduced plasma retinol level in diabetic rats, hepatic free retinol concentrations were decreased and the ratios of hepatic retinyl palmitate to retinol were increased without the alteration of hepatic retinyl palmitate concentrations in these animals. Meanwhile, the activities of CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase were decreased in the liver but not in the intestine in diabetic rats. These results suggest that the altered vitamin A status in the presence of diabetes is not only related to an inadequate availability of retinol carrier proteins as reported elsewhere (Tuitoek et al. 1996a;Lu et al. 2000), but also associated with the depressed REH activities in the liver of rats.

There is a substantial amount of evidence suggesting that several proteins critically involved in the metabolic availability of vitamin A are affected by one's vitamin A status. For example, the secretion of RBP from the liver into the circulation is inhibited if vitamin A is deficient (Blomhoff et al. 1991;Noy 2000). The principal enzymatic activity responsible for retinol esterification in the liver, LRAT, and its mRNA expression is almost undetectable in vitamin A-deficient rats, while retinol treatment rapidly recover LRAT activities in these animals (Randolph and Ross 1991;Zolfaghari and Ross 2000). A vitamin A deficient diet has also been shown to significantly reduce non-specific carboxylesterase activities in the hepatic cells of rats (Gad 1994). The vitamin A status, however, does not seem to have any influence on the hepatic activities of neutral and acid bile salt-independent REH (Matsuura et al. 1997). So far little is known about the effect of vitamin A on CHAPS-stimulated REH activity in the liver.

The reduced hepatic REH activities accompanied by decreased levels of plasma retinol in diabetic rats raises the question about whether REH activity responds to dietary vitamin A intake in diabetes. This study was therefore undertaken to examine the modifying effect of a diet containing either marginal, adequate, or excessive quantities of vitamin A on vitamin A metabolic status and the REH activities in diabetic BB rats.

4.2 Materials and methods

Materials

The chemical reagents were from Sigma-Aldrich Canada Limited (Oakville, Ontario, Canada) and Fisher Scientific Canada (Nepean, Ontario, Canada) unless otherwise stated.

Animals and diets

The animal protocol was reviewed and approved by the same committee described in chapter 2. The source of animals was also described earlier (chapter 2).

Thirty weanling male BBdp rats and the same number of age-matched male BBn rats were housed individually in our animal facility in stainless steel, wire-bottom cages under conventional conditions, as mentioned earlier in previous chapters. The rats were randomly assigned to one of the two diet groups and fed an open-formula, nonpurified NIH-07 basal diet modified to contain 0.81 or 15.61 mg RE (added as retinyl acetate) per kilogram of diet. These diets were designated low vitamin A (LVA) diet and high vitamin A (HVA) diet, respectively, and contained about 0.3 and 6 times as much vitamin A in NIH-07 basal diet. The vitamin A content of the NIH-07 basal diet was 2.61 mg RE/kg diet.

There were 14 BBdp rats and the same number of age-matched BBn control rats in the LVA diet group, and 16 BBdp and BBn rats in the HVA diet group. The rats consumed food and tap water ad libitum. Food intake was measured every other day and body weight was recorded weekly. Starting at 50~52 d of age, a plastic container was placed underneath a spout on the bottom of each cage to collect urine. Urine samples from BBdp rats were tested 3 times/wk for glucose level using Chemstrip uG (Boehringer Mannheim, Laval, Quebec, Canada) until the onset of diabetes, or until the rats reached the age of 120 d. When glucose higher than 56 mmol/L was detected in urine sample, blood glucose from the tail vein was measured in the fed state using a glucometer (Ames Miles, Toronto, Ontario, Canada). The onset of diabetes was defined as the time when blood glucose greater than 13.8 mmol/L in the fed state was detected (Hosszufalusi et al. 1993). BBdp rats at the onset of diabetes were described as BBd rats.

Each newly diagnosed BBd rat, along with its age-matched BBn control rat was terminated within 1 to 2 d of diabetes onset. All BBdp rats that did not develop diabetes and corresponding control animals were terminated at 120 d of age.

Blood and tissue collection

The rats were anesthetized with 5% halothane (Halocarbon Laboratories, River Edge, New Jersey, USA) after overnight food deprivation. A blood sample was removed via cardiac puncture and collected with a heparinized tube before rats were terminated via cervical dislocation. Plasma was later separated by centrifugation at 800 g for 20 min at 4°C and stored at –70°C until analyzed.

After the rats were terminated, the livers were quickly removed, rinsed with cold saline (4°C), blotted, weighed, and cut into small pieces. Liver microsomes were prepared immediately after the tissue collection on the same day and were used later for non-specific carboxylesterase analysis (see chapter 3 for detailed method of microsomal preparation). The remaining liver samples were quickly put into separate plastic vials and frozen immediately in liquid nitrogen before being stored at –70°C for subsequent analysis.

Vitamin A and E determination

Plasma and hepatic free retinol, α-tocopherol, and hepatic retinyl palmitate were extracted without saponification and determined by reverse-phase HPLC according to the methods of Tuitoek et al. (1996b), Wang et al. (1998) and Vliet (1991) as described in chapter 2.

A reverse phase Varian 5000 HPLC system (Varian Canada Ltd., Mississauga, Ontario, Canada) and a 5 μm Supelcosil LC-18 column (0.46 × 15 cm; Supelco, Ontario, Canada) with a C_{18} guard column were used to separate the vitamins. A Waters 486 tunable absorbance detector (Waters Chromatography Division, Millipore, Milford, Massachusetts, USA) was set at 325 nm for retinol and retinyl esters. A Shimadza RF-535 fluorescence HPLC monitor (Shimadza Corporation, Japan) providing excitation at 295 nm and emission at 330 nm was used to identify α-tocopherol.

The mobile phase used for the plasma samples was methanol-water (95: 5, v/v) at a flow rate of 1.5 ml/min. Retention time for retinol was about 3.2 min and α-tocopherol was about 10.2 min. The mobile phase used for the liver samples consisted of acetonitrile-tetrahydrofuran-water (solvent A: 50:20:30; solvent B: 50:44:6, v/v/v, with 1% (w/v) ammonium acetate and 0.35% (v/v) acetic acid in water) at a flow rate of 1 ml/min. The gradient procedure for the mobile phase for liver sample analysis was described in chapter 2. Retention time for retinol was 7.9 min and retinyl palmitate was 19.8 min. Retention time for α-tocopherol was 17 min. All runs were performed at ambient temperature.

Glucose, lipids analysis

Plasma glucose level was measured by the glucose oxidase method using Sigma kit (Procedure No. 315). Plasma triglyceride and total cholesterol levels were measured using enzymatic kits from Sigma (Procedure No. 336 and 401). Plasma HDL-cholesterol was determined by the same kit for total cholesterol measurement in the supernatant of plasma sample after VLDL and LDL were precipitated using HDL cholesterol reagent from Sigma (Catalog No. 352-4).

Liver triglyceride, total cholesterol and cholesterol ester were measured by the enzymatic methods adapted from Carr et al. (1993), Omodeo-Salè et al. (1984) and Radin (1981), as described in chapter 2.

All assays were performed in 96-well microtiter plates and the plates were read on a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA).

Hepatic function test

The L-alanine: 2-oxoglutarate aminotransferse (ALT) and the aspartate aminotransferase (AST) in plasma were measured using Sigma kits (procedure No. 152-UV and 122-UV, respectively) as the indicators of hepatic function in the rats. The kinetic assays of ALT and AST were performed in 96-well microtiter plates. The changes in absorbance per min were read at 340 nm on a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA). ALT and AST activities were expressed as international units per liter of plasma (U/L).

REH activities

The bile salt analog CHAPS-stimulated REH activity was measured in the liver according to the method of Cooper et al (1987;1990), as described in chapter 3.

Briefly, an aliquot of liver homogenates (about 300 μg of protein) was incubated in 100 mmol/L Tris-maleate buffer (pH 7.5) in a shaking water bath at 37°C for 90 min. The incubation mixture contained 200 mmol/L CHAPS in 100 mmol/L Tris-maleate and 1.5 mmol/L retinyl palmitate substrate with a final volume of 0.2 ml. The reaction was stopped by the addition of 0.4 ml of ethanol after the incubation period. Retinol in the incubation was then extracted and measured following the same HPLC procedure for liver vitamin A determination. CHAPS-stimulated REH activity was calculated from the amount of retinol produced during the assay and expressed as the nanomoles of retinol liberated per milligram of protein per h.

Non-specific carboxylesterase activities in the liver were measured by the method adapted from Chanda et al. (1997) and Zhang & Fariss (2002), as described in chapter 3. Hepatic microsomes were promptly prepared after the livers were collected from rats using differential centrifugation according to the method from Ong et al. (1987;1988). The enzyme assay was performed in 96-well, flat-bottom polystyrene microtiter plates using Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA). The non-specific carboxylesterase activity was then determined at 26°C by measuring the formation of *p*-nitrophenol (PNP) from *p*-nitrophenyl acetate as substrate. Non-specific carboxylesterase activity was calculated from the amount of *p*-nitrophenol produced during the assay and expressed as the nanomoles of *p*-nitrophenol formed per microgram of protein per min.

Statistical analysis

In order to examine the effect of diets on vitamin A metabolic status, REH activity and other biochemical indices in BB rats, data from BBd and BBn rats fed a NIH-07 basal diet containing 2.61 mg RE/kg diet as reported in chapter 3 were compared to the data from rats fed either a LVA diet (which contained 0.81 mg RE/kg diet) or a HVA diet (which contained 15.61 mg RE/kg diet) in this study.

All statistical analyses were conducted using the SAS system (SAS Institute, Cary, North Carolina, USA). Results were expressed as mean values with their standard errors. The main effects of diets (low vitamin A diet, basal diet and high vitamin A diet) and disease (diabetes and non-diabetes) and their interaction were assessed with a two-way ANOVA. Differences between groups were identified by least squares means. A two-tailed P-value of ≤ 0.05 was considered significant.

4.3 Results

General characteristics

The overall health of BB rats fed either a low or a high vitamin A diet was good. In rats fed a LVA diet, six out of fourteen (42.9%) BBdp rats developed diabetes by the end of the experiment, i.e., when rats reached the age of 120 d (Figure 4-1). The average diabetic onset age was 86 d with the earliest and latest age at 55 and 111 d, respectively. In the HVA diet group, five out of sixteen (31.3%) BBdp rats developed diabetes by the end of the experiment. The average onset age was 86 d with the earlist at 61 d and latest at 116 d. All the diabetic BB rats and their age-matched normal controls were killed within 1 or 2 d of diabetic onset.

The diabetic BB rats, either in a LVA diet group or in a HVA diet group, had significantly higher plasma glucose levels and 24 h urine excretion volumes than the age-matched BBn rats (Table 4-1). There were no differences in average daily food intake, final body weights and liver weights between BBd and BBn rats fed either LVA or HVA diet. Also, no significant differences in the above indices were found in BBd or BBn rats by diet groups.

The hepatic function tests including ALT and AST activities in the circulation were not affected by different diets, or by the presence of diabetes in the rats (Figure 4-2).

Plasma and hepatic lipid levels

Plasma triglyceride levels were dose-dependently increased with increased dietary vitamin A intake in both BBd and BBn rats (Figure 4-3 A). No influence of diabetes on plasma triglyceride levels was found irrespective of dietary vitamin A. Unlike the change of triglyceride, the plasma total cholesterol levels were significantly decreased with high vitamin A intake compared to rats with low or basal vitamin A intake in diabetic BB rats. In BBn rats, however, the plasma total cholesterol levels remained unaffected despite the different levels of dietary vitamin A (Figure 4-3 B). Similar changes were observed in plasma HDL-cholesterol levels in the rats (Figure 4-3 C).

Variation in the dietary vitamin A intake did not have any influence on the hepatic triglyceride concentrations in both BBn and BBd rats (Figure 4-4 A). The vitamin A intake, however, altered the hepatic levels of total and free cholesterol, but only in BBd rats (Figure 4-4 B, C). The cholesterol status was significantly increased in BBd rats fed a diet containing either a moderate (basal diet) or a high (HVA diet) level of vitamin A compared to that of the rats fed a LVA diet.

Plasma and hepatic vitamin A, E

At the onset of diabetes, the diabetic BB rats displayed significantly lower levels of plasma retinol compared to their non-diabetes counterparts (Figure 4-5). The depressed retinol levels remained unaltered irrespective of different amounts of dietary vitamin A intake. The variation of vitamin A in the diet also failed to alter the plasma levels of retinol in BBn rats.

Concentrations of retinol in the free or esterified form (as retinyl palmitate) were markedly lower in all BB rats (e.g. BBn and BBd rats) fed a LVA diet compared to the corresponding rats fed a basal or a HVA diet (Figure 4-6). The hepatic free retinols level remained similar in BBn rats fed either a basal diet or a diet supplemented with vitamin A. Similarly, no significant difference was found in hepatic free retinol levels in rats fed a high vitamin A diet compared to the same strain of rats fed a basal diet. When compared to their corresponding non-diabetes animals, the decreased hepatic free retinol levels in BBd rats fed a basal diet or a LVA diet were not observed in rats fed a HVA diet (Figure 4-6 A).

Unlike free retinol levels in the liver, the hepatic retinyl palmitate levels responded to vitamin A intake in a dose-related fashion (Figure 4-6 B). This trend was consistent in both BBd and BBn rats. No difference was observed between diabetic rats and their corresponding non-diabetes counterparts irrespective of the dietary vitamin A intake.

The plasma α-tocopherol levels were significantly elevated in diabetic BB rats compared to that of their corresponding normal counterparts. This was true irrespective of their vitamin A intake (Figure 4-7 A). BBd or BBn rats fed a diet containing high vitamin A, however, showed a reduction in plasma vitamin E levels compared to the rats fed a LVA diet.

Similar to the change of plasma α-tocopherol levels, the hepatic α-tocopherol concentrations were significantly higher in diabetic rats compared to those of controls fed either a LVA diet or a basal diet (Figure 4-7 B). However no difference was observed in hepatic α-tocopherol levels between BBd and BBn rats fed a HVA diet.

REH activities

Compared with BBn rats, the hepatic CHAPS-stimulated REH activities were significantly reduced in BBd rats (Figure 4-8). Variation in the level of vitamin A intake had no influence on this enzyme activity in either BBd or BBn rats.

Contrary to the reduced activity of hepatic non-specific carboxylesterase in diabetic BB rats fed a basal diet, this enzyme activity did not differ between BBd and BBn rats fed either a low or a high vitamin A diet (Figure 4-8). No effect of dietary vitamin A intake on the enzyme activity was observed in either BBd or BBn rats.

4.4 Discussion

This study was designed to investigate the modifying effect of low and high vitamin A intake on the activities of hepatic REHs, which are required for the hydrolysis of retinyl ester in vitamin A metabolism. In the liver, retinyl ester hydrolysis is an important reaction by which free retinol becomes available from its hepatic store (as retinyl esters). The influence of vitamin A intake on the retinyl ester hydrolysis in the presence of diabetes has not been properly studied before. This study has an important implication since the metabolic availability of retinol has been reported to be impaired in type 1 diabetes (Basu et al. 1989;Krempf et al. 1991;Martinoli et al. 1993). This was also found in our study of BBd rats (see previous chapters).

In the present study, weanling BB rats were given either a LVA diet (contained 0.81 mg RE/kg diet), a basal diet (contained 2.61 mg RE/kg diet), or a HVA diet (contained 15.61 mg RE/kg diet). The amounts of dietary vitamin A were chosen to better reflect vitamin A consumption in human population, which could be either marginally low or relatively high in intake of the vitamin concerned. That differs from the unusual practice of comparing the effects of very high and very low intake in some animal studies. Rats in this study fed the LVA diet were not manipulated to become vitamin A deficient animals. Rather, those rats were given enough vitamin A in the LVA diet for their normal maintenance, growth, and reproduction based on kinetic study (National Research Council (U.S.).Subcommittee on Laboratory Animal Nutrition. 1995). The rats fed a LVA diet were generally healthy in terms of body weight gains and daily food intake. It has been reported previously that rats fed a vitamin A-marginal diet containing only 0.35 mg RE per kilogram of diet still grew normally without resulting in significant liver storage of vitamin A (Dawson et al. 1999;Dawson et al. 2000). Meanwhile, physically normal rats supplemented with vitamin A to a level as high as 50 mg RE/kg diet for 8-10 months did not show any sign of vitamin A excess-associated hepatocellular damage, such as elevated plasma AST or bilirubin levels, hypoalbuminemia or hepatomegaly (Dawson et al. 1999). In the present study, there was no physical or biochemical evidence of vitamin A toxicity in any of the rats fed the HVA diet: rates of growth of those rats were normal, as were hepatic function tests such as ALT and AST activities in the rats.

An earlier study found that BB rats fed a retinol deficient diet had a much lower diabetic incidence (14% *vs* 40%) than rats fed a diet containing a common amount of vitamin A (4 mg retinyl palmitate per kilogram of diet) (Driscoll et al. 1996). The authors suggested that retinol, or a specific metabolite of retinol, is required for diabetogenesis in BB rats. In this study, the vitamin A content in a HVA diet was 20-fold higher than that in a LVA diet; the incidence of diabetes in rats fed a HVA diet was 31.3% compared to 42.9% in rats that received a LVA diet. No vitamin A dose-dependent effect on diabetes incidence was observed in this study. Although the sample size in the present study is rather small, our results provide no evidence supporting the aforementioned hypothesis, or at least, suggest that the influence of vitamin A in the pathogenesis of diabetes appears not to be dose-related. Our results are supported by another study in which retinyl palmitate, either given intraperitoneally or intravenously, protected against both STZ- and alloxan-induced diabetes in the rats (Chertow et al. 1989). However, it is also possible that complete vitamin A deficiency such as that reported in Driscoll's study (1996) could impair the immune function in the animals. That may delay or even prevent the development of this autoimmune disease in the rats.

It has been well documented that the plasma retinol level is maintained within a normal range of concentration as long as there is some minimal level of vitamin A in the liver or in extra-hepatic tissues (Blomhoff et al. 1991). In this study, BB rats were able to maintain a relatively stably plasma retinol level over the range of varying dietary vitamin A intake. Both BBd rats and BBn rats fed a LVA diet had very low vitamin A stores (as evidenced by low retinyl palmitate levels) and free retinol concentrations in the liver; however the plasma retinol levels were comparable to those in rats fed a basal or a HVA diet. Meanwhile, diabetic BB rats fed a HVA diet had far more vitamin A in their livers. The hepatic retinyl palmitate levels in rats fed a HVA diet were about 26-fold and 6-fold higher than those in BBd rats fed a LVA diet or a basal diet, respectively. The hepatic free retinol levels were also significantly elevated in rats fed a high vitamin A diet compared to rats fed a low vitamin A diet. However, no difference in hepatic free retinol was observed in rats fed either a basal or high vitamin A diet. The plasma concentrations of retinol in these rats were similar regardless of their dietary vitamin A content. These results indicate that the vitamin A mobilization in the liver is still depressed in diabetic BB rats despite feeding a diet containing high vitamin A. Vitamin A supplementation, at least at the current level, could not restore the reduced plasma retinol level except increase the hepatic vitamin A store in diabetic BB rats.

As noted earlier, two important proteins, REH and RBP, are involved in the mobilization of retinol from the storage pools of vitamin A in the liver. REH catalyzes the hydrolysis of retinyl ester in the liver to liberate retinol so the freed retinol is available to bind with RBP and enter the circulation. Decreased RBP levels in the plasma have been reported in BB rats with low plasma retinol levels (Lu et al. 2000). Our previous study found decreased enzymatic activities of REH in the liver of diabetic BB rats that suggests the alteration of the enzyme activities contribute to the decreased plasma retinol levels in the presence of diabetes in BB rats (chapter 3). The inhibition of REH activity in the liver could reduce the retinol availability to be bound to RBP. Meanwhile, the lower RBP level in the liver would lead to less retinol-RBP complex being formed and transferred into the circulation. Jing et al (2000) have reported that vitamin A supplementation (18 mg RE/kg diet) in diabetic BB rats did not bring the plasma retinol and RBP concentrations up to the levels in non- diabetic control animals. In this study, it was shown that the enzymatic activities of hepatic CHAPS-stimulated REH were reduced in the presence of diabetes in BB rats irrespective of dietary vitamin A intake. Meanwhile, the CHAPS-stimulated REH activities remained comparable in BBd rats fed three different levels of dietary vitamin A. This was also true in normal BBn control animals. These results clearly indicate that the hepatic CHAPS-stimulated REH activities in BB rats do not respond to a change of dietary vitamin A, at least under the current dietary vitamin A levels.

In agreement with the change of CHAPS-stimulated REH activity in the liver, this study has not revealed any significant alteration in hepatic carboxylesterase activity due to different dietary vitamin A intakes in both diabetic rats and non-diabetic control rats. These results do not agree with an earlier study. In that study, markedly reduced hepatic non-specific carboxylesterase activities were found in rats fed a diet containing no vitamin A, or marginal vitamin A level (0.18 μg vitamin A/g diet) compared to vitamin A sufficient rats fed a diet containing 4.0 μg vitamin A/g of diet (Gad 1994). It was postulated by the author that vitamin A might be a positive inducer of hepatic non-specific carboxylesterase synthesis. However, inconsistent with that hypothesis, the same study also reported that the administration of retinoic acid (the most potent regulator of gene expression among retinoids) failed to show any replenishment in hepatic carboxylesterase activities in the rats.

The lack of response of both hepatic CHAPS-stimulated REH activities and non-specific carboxylesterase activities to dietary vitamin A in diabetic BB rats as well as in normal control rats could be explained by the possibility that these two hepatic enzymes in BB rats is not altered by dietary vitamin A status at all. Or, the effect of dietary vitamin A on these enzyme activities in the liver would not appear at the young age of diabetic onset when BB rats were killed. Alternatively, it may be related to the fact that the difference in dietary vitamin A content among diet groups in our study was less dramatic. The hepatic vitamin A store in BB rats was never totally depleted, whereas in other studies pups were usually weaned from vitamin A deficient-diet-fed dams to eliminate hepatic vitamin A stores followed by a no-vitamin A diet to induce completely vitamin A deficiency in rats (Gad 1994;Randolph and Ross 1991).

Besides the increased hepatic vitamin A stores in both diabetic rats and control rats fed a HVA diet, higher triglyceride levels in the plasma but not in the liver in those rats were observed in this study. Meanwhile, the diabetic rats fed a HVA diet showed significantly decreased plasma total cholesterol and HDL-cholesterol concentrations compared to rats fed a basal or LVA diet. The hepatic total- and free cholesterol levels in BBd rats on a HVA diet were elevated. These results are in agreement with previous studies in which high doses of vitamin A significantly increased plasma triglyceride and LDL-cholesterol levels and decreased HDL-cholesterol concentrations in both humans and animals (Solomon and Erdman, Jr. 1980;Infante et al. 1991;Pastorino et al. 1991;Murray et al. 1983;Dawson et al. 1999). A subgroup of the Carotene and Retinol Efficacy Trial (CARET) participants showed a small, but not significant, increase in serum triglyceride levels when they were on the intervention and a decrease in serum triglyeride levels after the intervention (Redlich et al. 1999). Meanwhile, it was reported that CARET participants had a markedly increased cardiovascular mortality (by 26%) with long-term intake of β-carotene and vitamin A (as retinyl palmitate) compared to a placebo (Omenn et al. 1996). It is therefore possible that the increased triglyceride level and decreased HDL-cholesterol level observed after vitamin A supplementation may contribute to an increased risk of cardiovascular disease. Dyslipidemia is also associated with the pathogenesis of cardiovascular disease and other long-term complications in diabetic patients.

The present study found decreased plasma α-tocopherol levels along with increased dietary vitamin A content in both diabetic and non-diabetic rats. However, the plasma α-tocopherol levels were still higher in diabetic rats compared with their normal counterparts. Previous studies have suggested that a high dietary vitamin A intake decreases plasma vitamin E levels by increasing dietary vitamin E oxidation prior to reaching the intestine, interfering with vitamin E intestinal absorption, or increasing vitamin E turnover in the animals (Sklan and Donoghue 1982;Combs, Jr. 1976). Decreased vitamin E status is associated with increased oxidative stress, elevated lipid peroxidation, lowered glutathione level, enhanced endothelial dysfunction, all of which are thought to be related to the development of diabetes and its various complications (Dhein et al. 2003;Lonn et al. 2002;Jain et al. 2000).

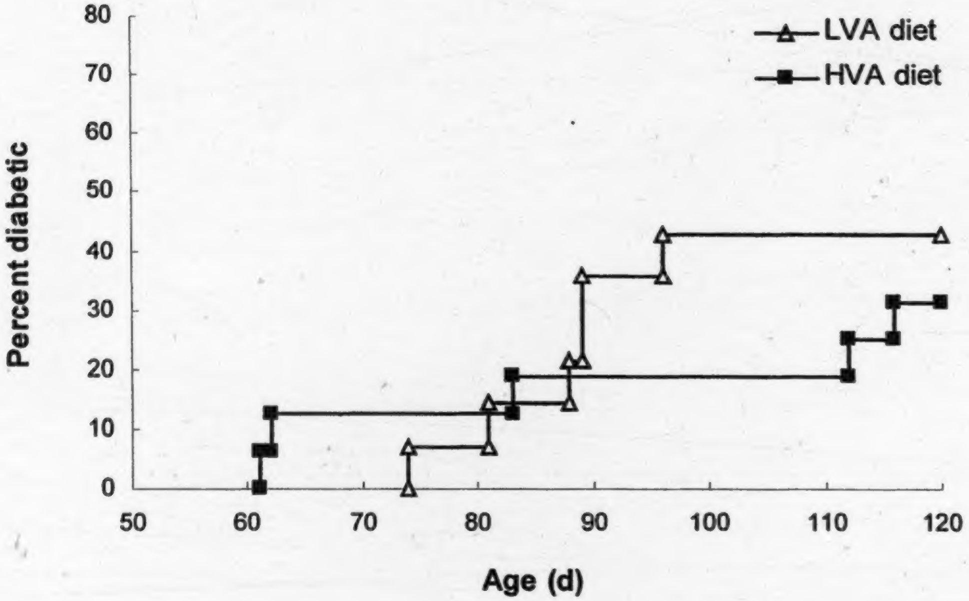


Figure 4-1 Cumulative diabetes incidence at age 120 d in BBdp rats fed a LVA or a HVA diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. In rats fed a LVA diet, six out of fourteen BBdp rats developed diabetes by the age of 120 d. The average onset age of diabetes in this group was 86.2 ± 3.1 d with the earliest at 55 d and the latest at 111 d. In rats fed a HVA diet, five out of sixteen BBdp rats developed diabetes by the age of 120 d. The average onset age of diabetes in these rats was 86.8 ± 11.8 d with the earliest at 61 d and the latest at 116 d.

Table 4-1 General characteristics and plasma glucose levels in BBd and BBn rats fed a LVA diet or a HVA diet[†][‡]

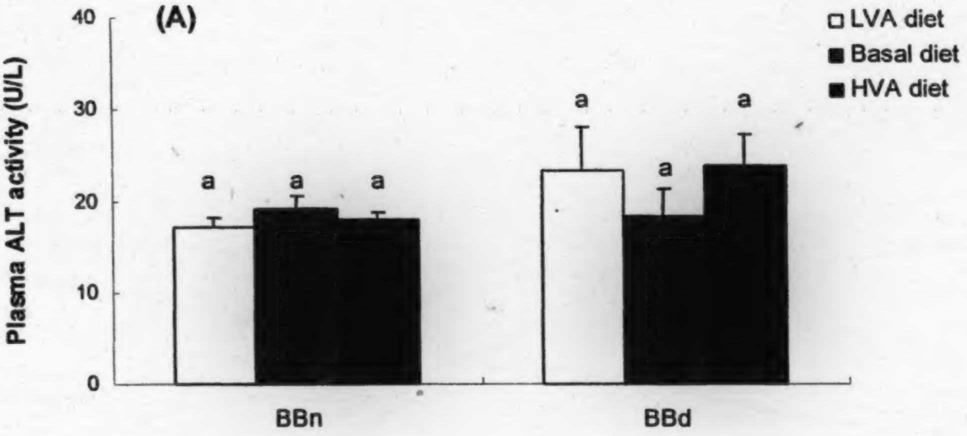
(Mean values with their standard error (SEM) for five or six rats per group)

	LVA diet				HVA diet			
	BBn		BBd		BBn		BBd	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Food intake (g/d)	18.99^{ab}	0.83	18.72^{b}	0.49	21.96^{a}	0.97	20.31^{ab}	1.52
Final body weight (g)	329.80^{a}	20.65	320.60^{a}	17.31	384.08^{a}	42.91	341.48^{a}	40.35
Liver weight (g)	11.41^{a}	0.80	12.04^{a}	0.68	13.41^{a}	1.08	12.10^{a}	1.22
24 h urine volume (ml/24 h)	6.60^{a}	1.41	74.00^{b}	10.26	5.66^{a}	1.08	82.61^{b}	13.38
Plasma glucose (mmol/L)	7.89^{a}	0.43	11.02^{b}	0.98	7.34^{a}	0.63	11.14^{b}	1.33

[†] BBn, non-diabetes-prone BB rats; BBd, diabetic BB rats. BBd and age-matched BBn rats were killed at the onset of diabetes.

[‡] The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet; the HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet.

Different superscript letters in a row indicate significant differences (two-way ANOVA with least squares means test, $P < 0.05$).



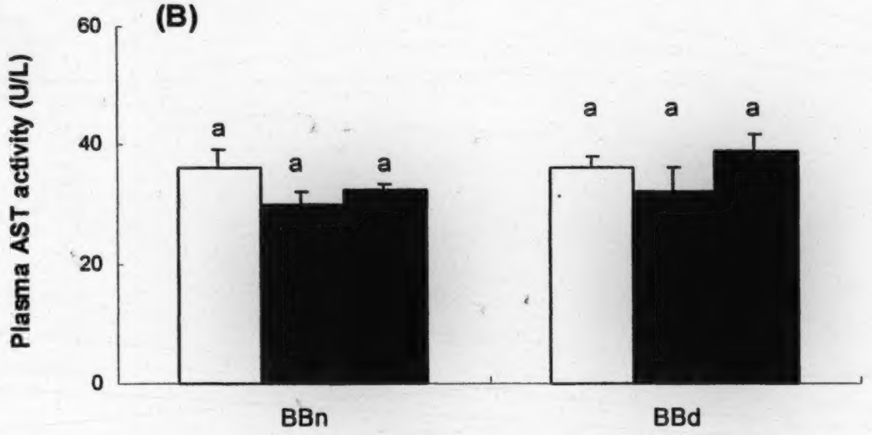


Figure 4-2 Plasma ALT (A) and AST (B) activities in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. No differences were found among BBd (or BBn) rats in different diet groups, or between BBd and BBn rats fed the same diet by two-way ANOVA ($P>0.05$).

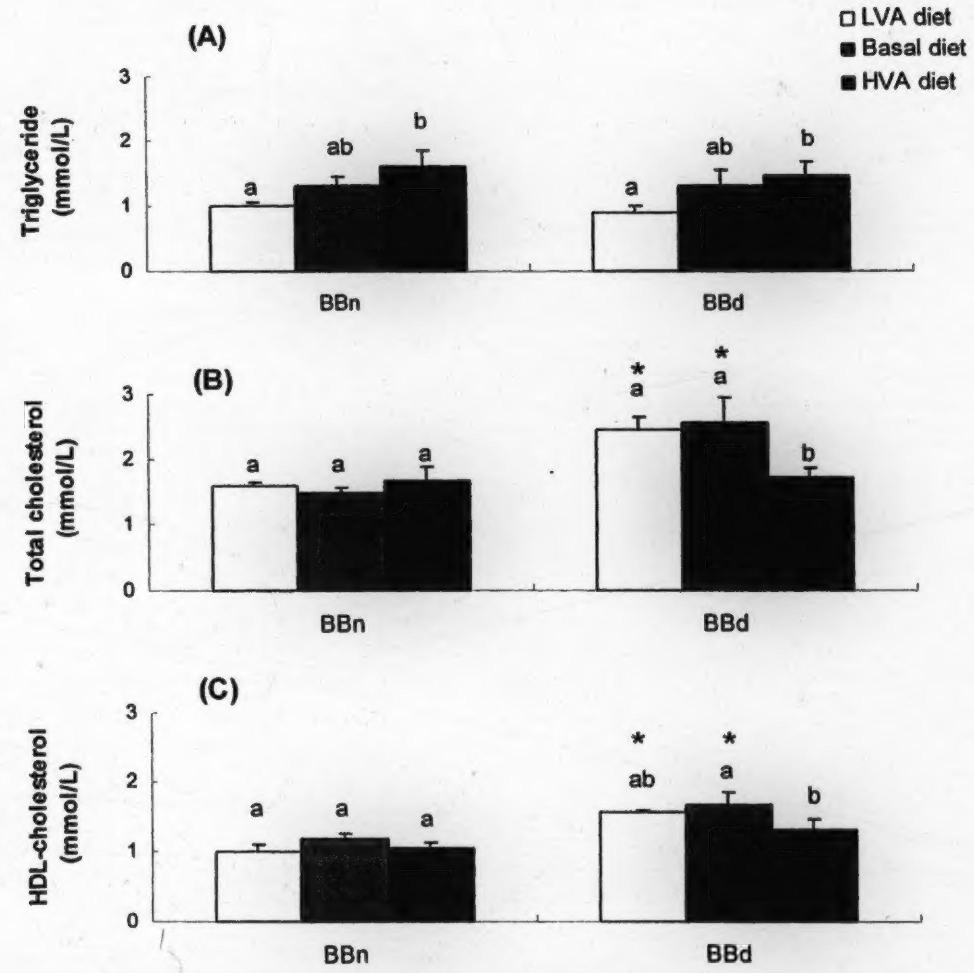


Figure 4-3 Plasma triglyceride (A), total cholesterol (B) and HDL-cholesterol (C) levels in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

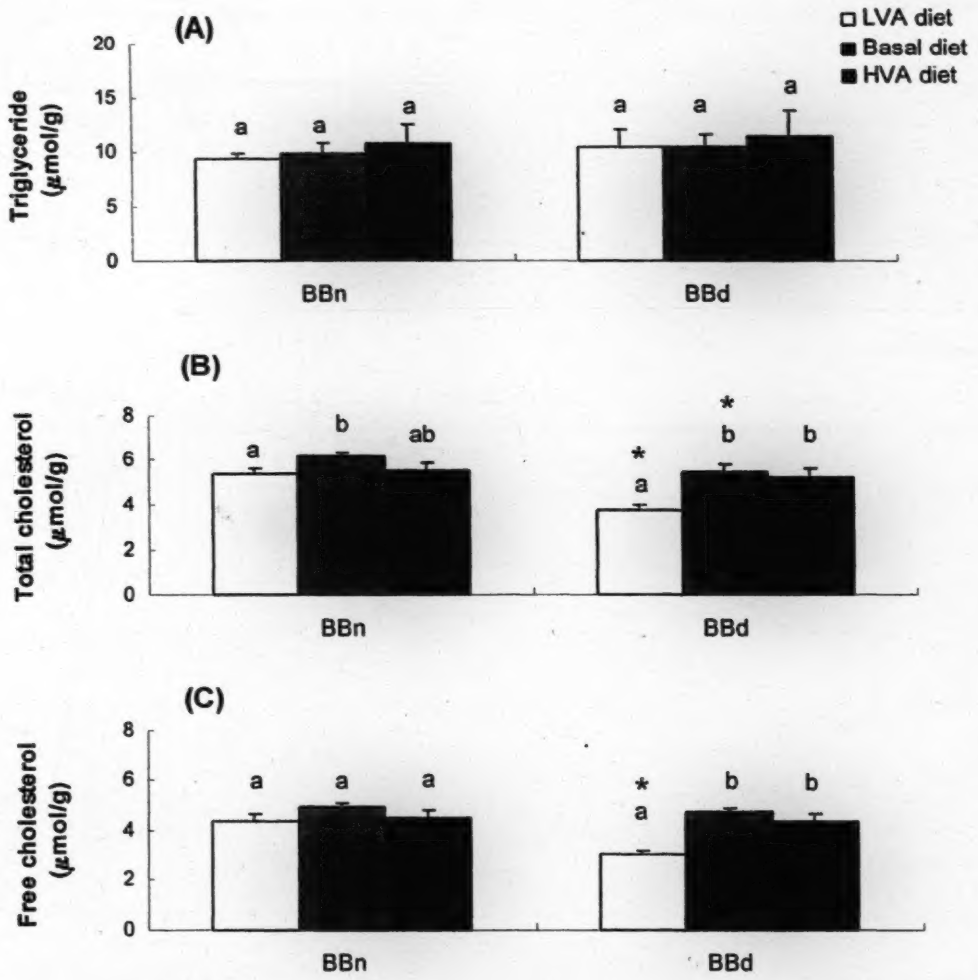


Figure 4-4 Hepatic triglyceride (A), total cholesterol (B) and free cholesterol (C) levels in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

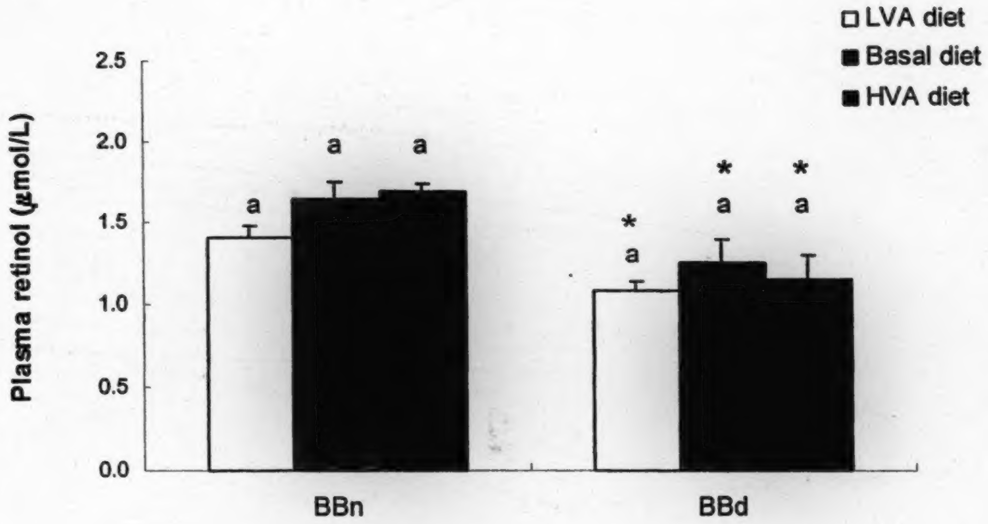


Figure 4-5 Plasma retinol levels in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

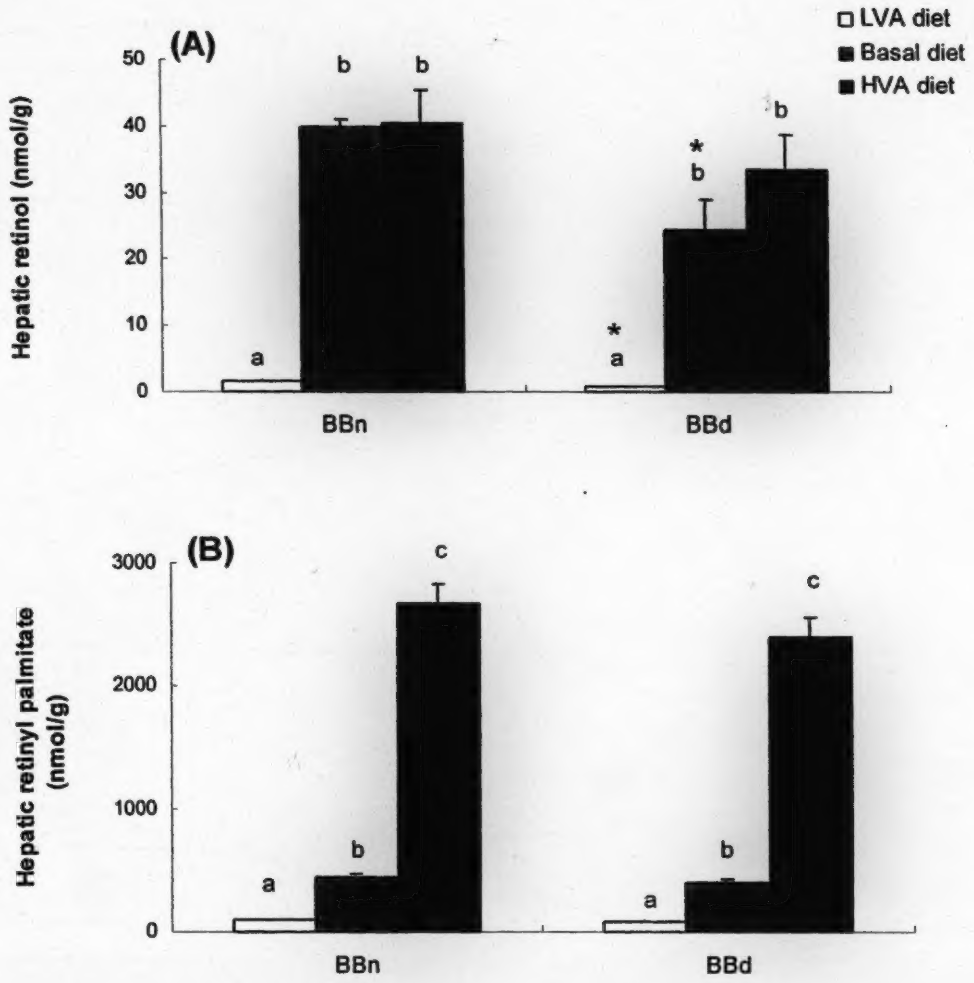


Figure 4-6 Hepatic retinol (A) and hepatic retinyl palmitate (B) levels in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

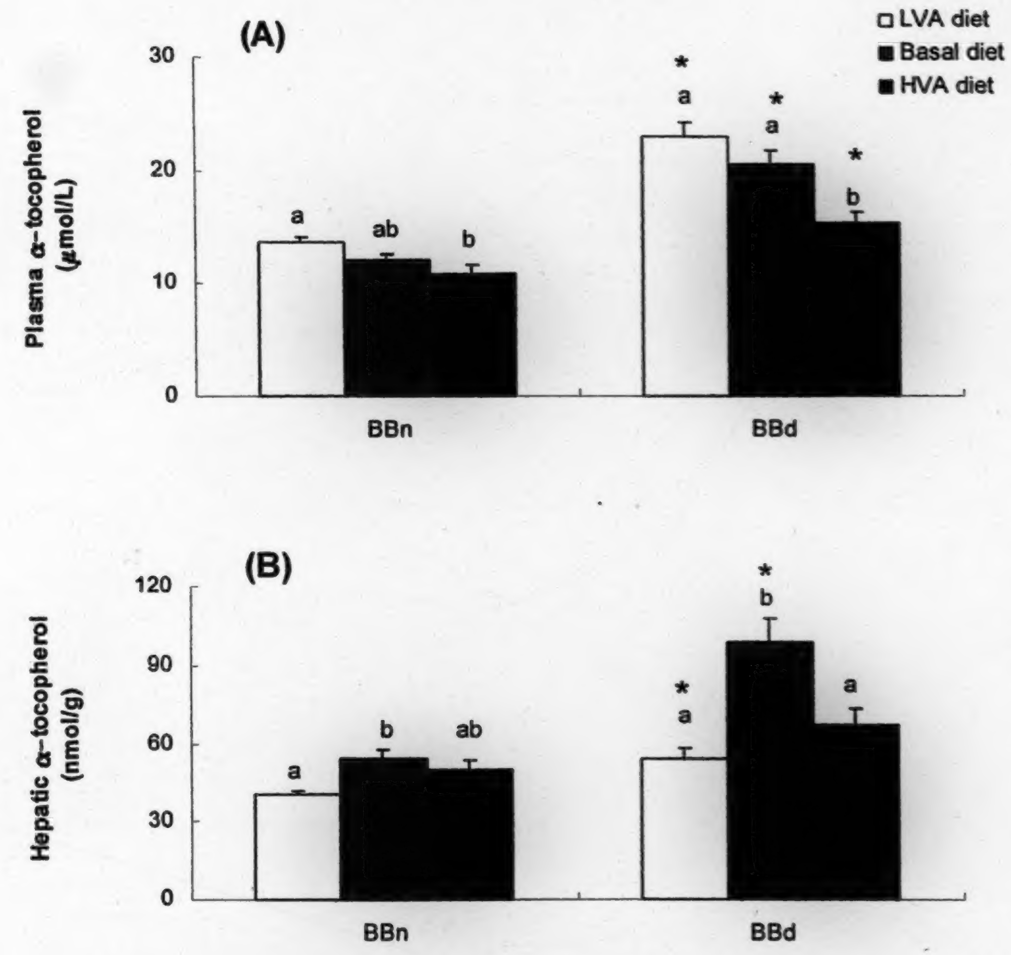


Figure 4-7 Plasma (A) and hepatic (B) α-tocopherol levels in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

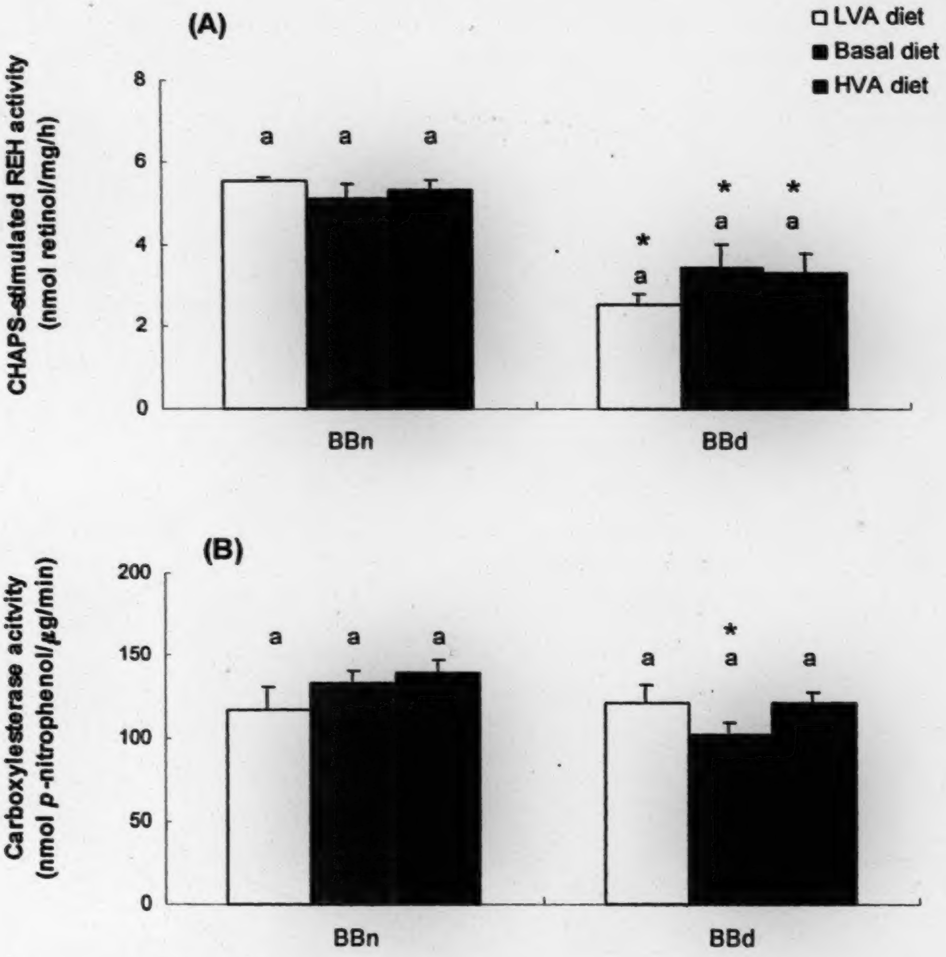


Figure 4-8 Hepatic CHAPS-stimulated REH (A) and non-specific carboxylesterase (B) activities in BBd and BBn rats fed a LVA diet, a HVA diet, or a basal diet.

The LVA diet (low vitamin A diet) contained 0.81 mg RE/kg diet. The HVA diet (high vitamin A diet) contained 15.61 mg RE/kg diet. The basal diet (NIH-07 diet) contained 2.61 mg RE/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values are means of five or six rats with their standard errors shown by vertical bars. Bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the diabetic rats and normal controls fed the same diet.

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Chapter 5

Dietary zinc improves hepatic vitamin A mobilization in BB rats

5.1 Introduction

Zinc is not only involved in vitamin A metabolism but is also associated with type 1 diabetes. Zinc deficiency is commonly linked to a low plasma vitamin A level despite adequate vitamin A content in a diet whereas the vitamin A status can be improved by zinc supplementation (Smith, Jr. et al. 1976;Ette et al. 1979;Brown et al. 1976). One of the explanations proposed for the relationship between zinc and vitamin A is that zinc deficiency is associated with a reduced hepatic synthesis of RBP, which facilitates the transportation of hepatic retinol to the circulation (Smith et al. 1974;Kelleher and Lönnerdal 2001). Zinc deficiency also affects intracellular transport of vitamin A by decreasing CRBP in the liver (Mobarhan et al. 1992). Conflicting results arose, however, when it was shown that RBP expression in the liver was induced by zinc deficiency both *in vitro* and *in vivo* (Satre et al. 2001;Kimball et al. 1995). Those results suggested that reduced plasma retinol level in zinc deficiency might be a direct consequence of low zinc level-related hormonal alterations and reduced food intake in the animals.

Apart from its association with the synthesis, storage and secretion of insulin in the pancreas (Zalewski et al. 1994), zinc has a number of insulin-like properties such as it enhances glucose uptake and glucose transport, and it stimulates lipogenesis (May and Contoreggi 1982). A previous study reported that zinc supplementation in BB rats significantly delayed the onset of overt diabetes, and also improved glucose tolerance in the animals (Tobia et al. 1998).

Increased urinary zinc excretion is commonly observed in both animal and human studies of type 1 diabetes (Lau and Failla 1984;Isbir et al. 1994;Brun et al. 1988;Heise et al. 1988). Some studies reported hyperzincuria-associated reduced plasma levels and tissue stores of zinc (Garg et al. 1994;Isbir et al. 1994;Chausmer 1998). In diabetic BB rats, hyperzincuria and decreased plasma zinc levels were reported in parallel with decreased levels of plasma retinol and RBP (Lu et al. 2000). It was therefore postulated by the authors that the disturbed zinc status in the presence of diabetes might contribute to decreased plasma RBP levels and retinol concentrations in rats (Lu et al. 2000).

Results from previous chapters of this thesis have shown that depressed hepatic REH activities co-existed with reduced hepatic and plasma retinol levels without alteration of hepatic retinyl ester concentrations (as retinyl palmitate) in diabetic BB rats. Those results suggest that depressed hepatic REH activities in diabetic rats may play a part in the altered vitamin A metabolic status by decreasing hepatic retinol mobilization, which limits hepatic free retinol availability. Zinc is known to be required for the activity of more than 300 enzymes including hepatic retinol (alcohol) dehydrogenase and retinal oxidase, two enzymes catalyzing hepatic retinol degradation to metabolites (McCall et al. 2000;Boron et al. 1988). However, it is unclear whether zinc is involved in the regulation of REH activity and thereby contributes to the limitation of hepatic free retinol availability in the presence of diabetes.

This study was therefore aimed to examine the possible change of REH activities as well as vitamin A metabolic status in diabetic BB rats supplemented with dietary zinc. The intestinal uptake of retinyl palmitate in the presence of zinc *in vitro* was also explored.

5.2 Materials and methods

Materials

The chemical reagents were from Sigma-Aldrich Canada Limited (Oakville, Ontario, Canada) and Fisher Scientific Canada (Nepean, Ontario, Canada) unless otherwise stated.

Animals and diets

The animal protocol was reviewed and approved by the same committee described in chapter 2. The source of animals was same as that described in chapter 2.

Sixteen weanling male BBdp rats and the same number of age-matched male BBn rats were housed individually in stainless steel, wire-bottom cages under conventional conditions, as described in chapter 2, in our animal facility. Rats were fed a NIH-07 based diet modified to contain 360 mg of elemental zinc (added as zinc sulfate) per kilogram of diet. This diet is refered to the zinc diet. Zinc content in NIH-07 basal diet was 60 mg/kg diet.

All rats had free access to food and tap water throughout the experiment. Food intakes were recorded every other day during the study, and all rats were weighed weekly. Beginning at age 50~52 d, a plastic container was placed underneath a spout on the bottom of each cage to collect urine. Urine samples from BBdp rats were tested 3 times/wk for glucose using Chemstrip uG

(Boehringer Mannheim, Laval, Quebec, Canada) until the onset of diabetes, or until the rats were 120 d old. Once the glucose concentration in the urine reached the level of 56 mmol/L, blood glucose from the tail vein was measured in the fed state using a glucometer (Ames Miles, Toronto, Ontario, Canada). The onset of diabetes was defined as the time when blood glucose of more than 13.8 mmol/L was detected in the fed state (Hosszufalusi et al. 1993). BBdp rats at the onset of diabetes were described as BBd rats.

Each newly diagnosed BBd rat, and its age-matched BBn control rat, was terminated within 2 d of diabetes onset. At age 120 d, all remaining BBdp rats that did not show glycosuria and hyperglycemia were terminated along with their control animals.

Urine, blood and tissue collection

Twenty-four hour urine samples were collected from each BBd rats and their age-matched BBn controls 1 d before termination. An aliquot of urinary sample was frozen for later analysis for urinary zinc content.

Before termination, the rats were deprived of food overnight and were anesthetized with 5% halothane (Halocarbon Laboratories, River Edge, New Jersey, USA). A blood sample was removed via cardiac puncture, and collected with a heparinized tube before the rats were terminated via cervical dislocation. An aliquot of whole blood (0.2 ml) was taken to collect erythrocyte on the same day. Plasma was separated from the remaining blood by centrifugation at 800 g for 20 min at 4°C and stored at –70°C.

After the rats were terminated, livers and kidneys were quickly removed, rinsed with ice-cold isotonic saline (4°C), blotted, weighed, and cut into small pieces. A portion of the liver samples was left on ice and used for microsomal preparation immediately after the tissue collection on the same day. Aliquots of tissue sample were quickly put into separate plastic vials and frozen immediately in liquid nitrogen before being stored at –70°C until analyzed.

Zinc determination

Tissue samples were prepared by a dry-ashing procedure prior to the determination of zinc content (Ihnat 2000). Liver and kidney (about 1 g) were weighed, put into crucibles, and dried in a muffle furnace (Blue M Electric Company, Blue Island, Illinois, USA) at 550°C overnight. The samples were removed from the furnace the next day and cooled at room temperature before adding 3 ml of 3 mol/L hydrochloric acid to the crucible and heating at 100°C for 10 min to dissolve the ash. The resulting samples were appropriately diluted with 0.1 mol/L hydrochloric acid and were then ready for determination of zinc (at 213.9 nm) using an atomic absorption spectrophotometer (Perkin Elmer 4000, Norwalk, Connecticut, USA). Zinc standard stock solution (1 mg/ml) was diluted with 0.1 mol/L hydrochloric acid before use.

Urine samples were added to 10% (w/w) of hydrochloric acid (5:1, v/v) and further diluted with water prior to the determination of zinc with an atomic absorption spectrophotometer (Kiilerich et al. 1980). The plasma zinc level was measured according to the procedure published by Association of Analytical Communities (AOAC) (2000). Plasma was first diluted with 5 volume of 0.03% Brij 35 solution and then zinc was determined with an atomic absorption spectrophotometer. The zinc concentrations in the liver, kidney, urine and plasma were also measured in BBd and age-matched BBn rats fed a NIH-07 basal diet (as described in chapter 3).

Superoxide dismutase analysis

The activity of a zinc-containing antioxidant enzyme, copper/zinc superoxide dismutase (Cu/Zn SOD), was measured in erythrocytes as another zinc-status indicator (Davis et al. 2000). Freshly collected whole blood (0.2 ml) was centrifuged at 800 g for 20 min at 4°C to separate plasma and erythrocytes. The erythrocytes were then washed four times with 1.5 ml of 0.9% NaCl solution and centrifuged at 800 g for 10 min after each wash. The washed and centrifuged erythrocytes were mixed with 0.5 ml of cold redistilled water and were left to stand at 4°C for 15 min to lyse. Hemolysates were stored in -70°C for subsequent Cu/Zn SOD analysis using the RANSOD kit from Randox (Randox Laboratories Canada Ltd., Mississauga, Ontario, Canada).

The Cu/Zn SOD activity was measured on the basis of its inhibitory action on the rate of superoxide-dependent reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride by xanthine and xanthine oxidase. Analysis was carried out on a Cary UV-Visible spectrophotometer (Varian Instruments, Sugar Land, Texas, USA) at 500 nm. Cu/Zn-SOD was expressed as the number of SOD units per gram of hemoglobin.

The hemoglobin concentration in hemolysates was measured colorimetrically by the cyanmethemoglobin method using Sigma kit (procedure No. 525).

Cu/Zn-SOD activity was also measured in BBd and age-matched BBn rats fed a NIH-07 basal diet (as described in chapter 3).

Vitamin A and E determinations

Plasma and hepatic free retinol, α-tocopherol, and hepatic retinyl palmitate were extracted without saponification and determined by reverse-phase HPLC according to the methods of Tuitoek et al. (1996), Wang et al. (1998) and Vliet (1991) as described in chapter 2.

Briefly, the retinoids and α-tocopherol were extracted from liver homogenates and plasma samples. An aliquot of extract was injected into a reverse phase Varian 5000 HPLC system (Varian Canada Ltd., Mississauga, Ontario, Canada) with a 5 μm Supelcosil LC-18 column (0.46 × 15 cm; Supelco, Ontario, Canada) and a C_{18} guard column to separate the vitamins. A Waters 486 tunable absorbance detector (Waters Chromatography Division, Millipore, Milford, MA) was set at 325 nm for the analysis of retinol and retinyl esters. A Shimadza RF-535 fluorescence HPLC monitor (Shimadza Corporation, Japan) provided excitation at 295 nm and emission at 330 nm was used to identify α-tocopherol.

The mobile phase used for the plasma samples was methanol-water (95: 5, v/v) at a flow rate of 1.5 ml/min. The mobile phase used for the liver samples consisted of acetonitrile-tetrahydrofuran-water (solvent A: 50:20:30; solvent B: 50:44:6, v/v/v, with 1% ammonium acetate and 0.35% acetic acid in water) at a flow rate of 1 ml/min. The gradient procedure was described in chapter 2.

Glucose, lipids analysis

Plasma glucose level was measured by the glucose oxidase method using Sigma kit (Procedure No. 315). Plasma triglyceride and total cholesterol levels were measured using enzymatic kits from Sigma (Procedure No. 336 and 401). Plasma HDL-cholesterol was determined by the same kit for total cholesterol measurement in the supernatant of plasma sample after VLDL and LDL were precipitated using HDL cholesterol reagent from Sigma (Catalog No. 352-4).

Hepatic levels of triglyceride, total cholesterol and cholesterol ester were measured by the enzymatic methods adapted from Carr et al. (1993), Omodeo-Salè et al. (1984) and Radin (1981), as described in chapter 2.

All assays were performed in 96-well microtiter plates and the plates were read on a Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA).

REH activities

CHAPS-stimulated REH activity was measured in the liver according to the method of Cooper et al (1987;1990), as described in chapter 3. Briefly, an aliquot of liver homogenate (about 300 μg of protein) was incubated with 200 mmol/L CHAPS and 1.5 mmol/L retinyl palmitate substrate in 100 mmol/L Tris-maleate buffer (pH 7.5) in a shaking water bath at 37°C for 90 min. The final volume in the incubation was 0.2 ml. The reaction was stopped by the addition of 0.4 ml of ethanol after the incubation period. Retinol in the incubation was then extracted and measured following the same HPLC procedure for hepatic vitamin A determination. CHAPS-stimulated REH activity was calculated from the amount of retinol produced during the assay and expressed as the nanomoles of retinol liberated per milligram of protein per h.

Non-specific carboxylesterase activities in the liver were measured by the method adapted from Chanda et al. (1997) and Zhang & Fariss (2002), as described in chapter 3. Hepatic microsomes were promptly prepared after the livers were collected from rats using differential centrifugation according to the method from Ong et al. (1987;1988). Non-specific carboxylesterase activity was then determined by measuring the formation of *p*-nitrophenol (PNP) from *p*-nitrophenyl acetate. The enzyme assay was performed in 96-well, flat-bottom polystyrene microtiter plates using Spectra Max 190 Microplate spectrophotometer (Sunnyvale, California, USA). Non-specific carboxylesterase activity was calculated from the amount of *p*-nitrophenol produced during the assay and expressed as the nanomoles of *p*-nitrophenol formed per microgram of protein per min.

Intestinal uptake of retinyl palmitate co-existing with zinc

The effect of zinc on the intestinal uptake of retinyl palmitate was determined following a procedure described by Tuitoek et al. (1994), as described in chapter 3. Briefly, one segment of proximal jejunum and distal ileum were rapidly removed from the rats promptly after their termination. The samples were opened along the mesenteric border and rinsed gently with cold saline. Circular disks of intestine were cut from the segment, mounted as flat sheets in incubation chambers, and pre-incubated in oxygenated Krebs buffer for 15 min at 37°C. The chambers were then transferred to beakers containing [carboxyl-^{14}C] inulin (NEN Life Science Products, Inc., Boston, Massachusetts, USA), and [15-^{3}H] retinyl palmitate (American Radiolabeled Chemicals, Inc., St Louis, Missouri, USA) at concentrations of 32 nmol/L and 256 nmol/L, respectively, with or without the existence of zinc at a concentration of 0.153 mmol/L (added as zinc sulfate). After incubation for 6 min at 37°C, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline. The exposed mucosal tissue was cut out of the chamber and placed on glass slides. The tissue was dried overnight at 55°C and the dry weight of each sample was determined. The dried sample was later saponified with 0.1 mol/L sodium hydroxide. The radioactivity of the sample was determined by means of an external standardization technique to correct for variable quenching of the two isotopes. The presence of any adherent mucosal fluid was assessed by the presence of the non-absorbable marker, [carboxyl-^{14}C] inulin, added in the incubation buffer.

The rate of uptake of retinyl palmitate was expressed as the picomoles of the retinyl palmitate taken up into the intestine per 100 mg dry weight of tissue per min (pmol/100 mg/ min).

Statistical analysis

In order to determine the effect of zinc supplementation on vitamin A metabolic status, REH activity and other biochemical indices in diabetic BB rats, data from BBd and BBn rats fed a NIH-07 basal diet (contained 60 mg zinc/kg diet) as reported in chapter 3 were used for comparison with data from rats fed a zinc diet (contained 360 mg zinc/kg diet) in this study. All statistical analyses were conducted using the SAS system (SAS Institute, Cary, North Carolina, USA). Results were expressed as mean values with their standard errors. The main effects of diets (basal and zinc diet) and disease (diabetes and non-diabetes) and their interactions were assessed with a two-way ANOVA. Differences between groups were identified by the least square means test. A two-tailed *P*-value of ≤ 0.05 was considered significant.

5.3 Results

General characteristics and zinc status

All BB rats receiving a zinc-enriched diet grew well. Eight out of the sixteen BBdp rats developed diabetes before the age of 120 d. The diabetic incidence in these rats was 50%. The earliest and latest onset ages were 55 and 111 d, respectively, with an average onset age at 76 d. Compared with their age-matched non-diabetic counterparts, BBd rats consumed similar amount of food daily. The final weight gains between the two groups of rats were comparable. The 24 h urinary excretion and plasma glucose levels in the BBd rats were as expectantly higher at the onset of diabetes than that in control rats (Table 5-1).

Zinc levels in the plasma, urine and the tissues, including the liver and the kidneys, were measured in BB rats fed a NIH-07 basal diet, or a zinc-supplemented diet. Neither the presence of diabetes nor the zinc-supplemented diet had an effect on the plasma zinc concentrations (Figure 5-1 A). Dietary zinc supplementation resulted in a markedly increased urinary excretion of zinc in 24 h in the diabetic rats but not in the non-diabetic rats (Figure 5-1 B). Similar to the content of urinary zinc, the zinc concentrations in the liver as well as in the kidneys were significantly increased in the diabetic rats fed a zinc-supplemented diet but not in BBd rats fed a basal diet (Figure 5-2 A and B).

The activity of erythrocyte Cu/Zn SOD was significantly increased in both BBn and BBd rats fed a diet supplemented with zinc (Figure 5-3). No difference was observed between diabetic rats and control counterparts fed the basal diet. Zinc-supplemented diet, however, markedly increased Cu/Zn SOD activity in the erythrocyte in the presence of diabetes.

Plasma and hepatic lipid levels

Neither zinc supplementation nor the presence of diabetes affected triglyceride levels in the plasma of BBd and BBn rats (Figure 5-4 A). It was, however, noteworthy that plasma total cholesterol levels were significantly decreased in diabetic BB rats fed a zinc-supplemented diet compared with BBd rats receiving a basal diet (Figure 5-4 B). However, no difference in plasma total cholesterol levels was found in BBn rats fed a basal diet either alone or supplemented with zinc. Diabetic BB rats had consistently higher plasma HDL-cholesterol levels compared to BBn control rats irrespective of dietary zinc supplementation (Figure 5-4 C).

Similar to the change in the plasma, hepatic triglyceride levels did not respond to either dietary zinc supplementation, or the presence of diabetes (Figure 5-5). Hepatic total and free cholesterol concentrations were significantly decreased in zinc-supplemented BBd rats compared to control rats fed the same diet, or BBd rats fed a basal diet. There was no significant effect of dietary zinc on hepatic total or free cholesterol levels in non-diabetic BB rats.

Vitamin A, E levels and REH activities

Diabetic BB rats, fed either a NIH-07 basal diet or zinc-supplemented diet, showed significantly decreased plasma retinol levels at the onset of diabetes compared with their corresponding age-matched BBn controls (Figure 5-6, A). Dietary zinc supplementation did not affect the plasma retinol level in these rats. However, hepatic retinol levels were elevated significantly after dietary zinc supplementation in both BBd and BBn rats. While retinol concentrations in the liver were decreased in the presence of diabetes in BB rats receiving a basal diet, the concentrations did not differ between BBd and BBn rats supplemented with dietary zinc (Figure 5-6, B).

Hepatic retinyl palmitate levels did not change with either dietary zinc supplementation, or presence of diabetes in BB rats (Figure 5-7, A). However, while the molar ratios of hepatic retinyl palmitate to retinol were significantly elevated in the presence of diabetes in rats fed a basal diet, the ratios did not differ in BBd and BBn rats fed the zinc-supplemented diet (Figure 5-7, B). When compared to the same strain of rats fed a basal diet, BBd rats on a diet containing added zinc had markedly reduced ratios of retinyl palmitate to retinol in the liver. This difference was not significant between BBn rats fed a basal diet and BBn rats fed a zinc-supplemented diet.

In the presence of diabetes, BBd rats had consistently higher α-tocopherol concentrations in both plasma and the liver compared to age-matched BBn controls with or without dietary zinc supplementation. Zinc supplementation did not affect plasma or hepatic α-tocopherol levels in either BBd or BBn rats (Figure 5-8).

The CHAPS-stimulated REH activities in the liver were significantly decreased in diabetic rats fed a basal diet or a zinc-supplemented diet when compared with corresponding age-matched non-diabetic counterparts. In both BBd and BBn rats, hepatic CHAPS-stimulated REH activity was not affected by dietary zinc supplementation (Figure 5-9, A).

The change of non-specific carboxylesterase activities in the liver was not consistent. It was decreased in the presence of diabetes in rats fed a basal diet, but increased in diabetic rats fed a zinc-supplemented diet (Figure 5-9, B). Diabetic BB rats had lower non-specific carboxylesterase activities than age-matched normal control rats fed a basal diet. However in the rats fed a zinc-supplemented diet the enzyme activities were higher in diabetic rats compared to BBn control rats.

Effect of zinc on intestinal uptake of retinyl palmitate *in vitro*

As presented in chapter 3, a linear relationship was observed between the concentrations of retinyl palmitate and its uptake rate into the jejunum and the ileum when the concentrations of retinyl palmitate were varied between 32 and 256 nmol/L. The concentrations retinyl palmitate used in the incubation are well within the physiological range reported in previous study (Hollander and Muralidhara 1977). With the addition of physiological concentration of zinc

(0.153 mmol/L) (Wapnir et al. 1989;Wapnir and Stiel 1986) in the incubation, the rate of retinyl palmitate uptake into both the jejunum and the ileum in diabetic BB rats was significantly elevated. The slope of the line between the rate of uptake and the concentration of retinyl palmitate was markedly increased in the existence of zinc in diabetic rats (15.4±1.6 *vs* 93.5±23.5 without and with zinc in the jejunum, and 23.9±4.5 *vs* 81.3±31.1 without and with zinc in the ileum; $P<0.05$). A similar trend was found in BBn rats (20.1±3.3 *vs* 73.3±21.7 without and with zinc in the jejunum, and 20.5±1.8 *vs* 54.6±25.8 without and with zinc in the ileum; $P<0.05$). There was no difference in the uptake of retinyl palmitate between the diabetic rats and control animals irrespective of the existence of zinc (Figure 5-10).

5.4 Discussion

The dietary zinc requirement in weanling and adult rats is estimated to be 12 to 18 mg/kg diet depending on the source of primary protein in the diet (National Research Council (U.S.).Subcommittee on Laboratory Animal Nutrition. 1995). Results from an earlier study showed that BB rats with dietary zinc supplementation as high as 1000 mg/kg diet grew well, consumed a normal amount of food and water, and exhibited no signs of zinc toxicity (Tobia et al. 1998). In that experiment the diabetic incidence in BB rats (19%) was reduced by almost 60% at 90 d of age compared to the rats receiving normal zinc diet or low zinc diet containing 50 or 1 mg zinc per kilogram of diet. In this study, the zinc content in the zinc-supplemented diet was 360 mg zinc per kilogram of diet, which is about 6-times the amount in the NIH-07 basal diet containing zinc 60 mg/kg diet. The incidence of diabetes was 50% in zinc-supplemented BB rats in the present study, and about 37-43% in BB rats fed a basal diet. No statistical comparison on diabetic incidence was made due to the small sample size in our study. However, zinc supplementation at the current level did not appear to delay or prevent the development of diabetes in BB rats.

Increased urinary zinc excretion and decreased plasma zinc levels have been reported in the presence of diabetes (Lau and Failla 1984;Isbir et al. 1994;Lu et al. 2000). The results reported here have not found any difference in urinary zinc contents, plasma zinc concentrations or the hepatic zinc levels in diabetic BB rats fed a NIH-07 basal diet compared to non-diabetic controls. The lack of change in urinary zinc excretion and in the plasma zinc level in diabetic rats found here might be explained by the fact that (1) zinc status is usually homeostatically regulated in the body. (2) the rats were killed only 1 or 2 d after the onset of diabetes in this study. Such a short period may not be long enough to cause an increase in urinary zinc excretion in diabetic rats. It should be mentioned that there is still no universally accepted single measure suitable to accurately determine the zinc status in the body. Plasma zinc concentration is insensitive to changes in dietary zinc and therefore is generally considered a poor measure of marginal zinc deficiency (Wood 2000). However, results in our study did show that zinc status in diabetic rat was altered to some extent, as indicated by increased renal zinc concentrations in the presence of diabetes. Moreover, urinary zinc excretions differed significantly between diabetic BB rats and their non-diabetic controls when the rats were fed a zinc-supplemented diet, indicating an altered urinary zinc excretion in the diabetic state. Meanwhile, zinc supplementation at the current level was able to improve the zinc status in diabetic BB rats, as shown by significantly increased zinc levels in tissues such as the liver and the kidneys, as well as increased erythrocyte Cu/Zn SOD activities in the presence of diabetes.

Results from this study suggest that hepatic vitamin A metabolism is affected by zinc supplementation in BB rats. The hepatic retinol levels in BBd rats receiving a zinc-supplemented diet were elevated by almost 105% compared to the BBd rats fed a basal diet. No modifying effect of zinc on hepatic retinyl palmitate levels was observed in the rats. The molar ratios of retinyl palmitate to retinol were markedly reduced (about 46%) with zinc supplementation in diabetic BB rats. Non-diabetic normal controls also showed increased hepatic free retinol levels with zinc supplementation although the increase in BBn rats (35% of that in rats fed a basal diet) was less than that in BBd rats. The molar ratios of retinyl palmitate to retinol did not differ between BBn rats fed different diets. These results indicate that more retinol is liberated from retinyl ester in the liver of a diabetic BB rat when dietary zinc is increased.

The results of this study show that hepatic REH activity was affected by dietary zinc supplementation. The hepatic non-specific carboxylesterase activities were increased significantly in diabetic rats fed a zinc-supplemented diet compared to either their age-matched non-diabetic controls on the same diet, or the BBd rats fed a basal diet. However, the decreased activities of hepatic CHAPS-stimulated REH could not be restored by zinc supplementation in BB rats in the presence of diabetes. These results were supported by another study, in which it was found that CHAPS-stimulated REH activities in the liver were not altered by zinc deficiency in healthy rats (Boron et al. 1988). A previous study also found that zinc effectively inhibited the neutral bile salt-independent REH activity but not the acid bile salt-independent REH activity *in* *vitro* (Gad and Harrison 1991). It is therefore possible that zinc participates in the regulation of some, but not all, REH activities in the liver.

The effect of zinc on some of other hepatic enzymes that regulate vitamin A metabolism in the rats has been reported previously. Rats receiving a low zinc diet of 2.3 mg zinc /kg diet for 3 wk showed a significant reduction in the activities of retinol (alcohol) dehydrogenase and a significant increase in the activities of retinal oxidase when compared with rats fed a control diet with zinc content of 50 mg/kg diet (Boron et al. 1988). No difference in the activities of microsomal acyl coenzyme A: retinol acyl transferase (ARAT) in the liver was detected in rats fed a low zinc diet. It would have been more meaningful if the hepatic lecithin: retinol acyl transferase (LRAT) activities had been examined in that study because LRAT is the enzyme responsible for the esterification of free retinol under physiological conditions while ARAT is involved in the esterification of retinol only when free retinol is presented in a large amount in the liver (Yost et al. 1988;Blomhoff et al. 1991).

Results presented here also show that the intestinal uptake of retinyl palmitate into the jejunum and the ileum was markedly increased by the co-existence of zinc irrespective of the presence of diabetes. Our results are supported by an earlier study in which it was observed that rats supplemented with a large dose of zinc for 10 d significantly increased the activities of hydrolase enzyme including amylase, lipase and trypsin in the small intestine and pancreas (Szabo et al. 2004). It is possible that zinc also increases REH activities in the intestine in rats.

Although vitamin A mobilization along with the non-specific carboxylesterase activities in the liver were increased after dietary zinc supplementation, dietary intervention in the present study failed to improve the depressed plasma vitamin A levels in diabetic rats. This is disappointing but is consistent with another study, in which the combination of zinc and vitamin A supplementation (180 mg of zinc and 18 mg RE per kilogram of diet) in BB rats did not increase either plasma retinol levels or RBP concentrations in diabetic rats while hepatic RBP gene expression was enhanced with zinc plus vitamin A supplementation in the presence of diabetes (Lu et al. 2000).

Zinc supplementation did not affect triglyceride levels in the plasma and the liver, whereas plasma and hepatic total cholesterol levels were significantly decreased in zinc-supplemented diabetic BB rats compared with rats receiving a basal diet. There was no significant influence of zinc supplementation on the plasma or hepatic lipids in non-diabetic rats. These results are in agreement with a previous study, which implied that zinc supplementation improved diabetes-related lipid metabolic disorder, including increased plasma triglyceride, total-cholesterol and LDL-cholesterol concentrations in rats (Wu et al. 2004). However, the HDL-cholesterol levels in the diabetic rats were increased by zinc supplementation (Wu et al. 2004). The response of lipids to zinc supplementation appears to differ between healthy and diabetic subjects. It has been reported that zinc supplementation significantly depresses the plasma HDL-cholesterol levels in healthy human subjects or in healthy rats (Cho et al. 1989;Black et al. 1988;Hooper et al. 1980). A study of a group of healthy elderly people found a significant increase in HDL-cholesterol levels after zinc supplementation was stopped (Goodwin et al. 1985). This HDL-cholesterol-lowing effect of zinc observed in those studies is not fully explained. Some studies suggest that this effect may be related to the lipid abnormality induced by low copper status due to an impaired copper absorption in the presence of excess dietary zinc (Klevay et al. 1984;Allen and Klevay 1978).

Table 5-1 General characteristics and plasma glucose levels in BBd and BBn rats fed a zinc diet [†] [‡]

(Mean values with their standard errors (SEM) for seven rats per group)

	BBn		BBd		Significance, *P**
	Mean	SEM	Mean	SEM	
Food intake (g/d)	20.25	0.94	19.75	0.98	NS
Final body weight (g)	343.1	34.2	284.8	39.4	NS
Liver weight (g)	12.20	1.10	10.51	1.08	NS
24 h urine volume (ml/24 h)	6.22	1.25	93.33	9.36	<0.0001
Plasma glucose (mmol/L)	7.34	0.69	17.22	2.18	<0.0001

† BBn rats, non-diabetes-prone BB rats; BBd rats, diabetic BB rats. BBd rats and age-matched BBn rats were killed at the time of diabetes onset. The average onset age of diabetes was 76.4± 7.9 d with the earliest at 55 d and the latest at 111 d.

‡ The Zinc diet contained 360 mg zinc/kg diet.

* Significant difference between BBd rats and age-matched normal BBn rats was determined by Student's *t*-test. NS = *P*>0.05.

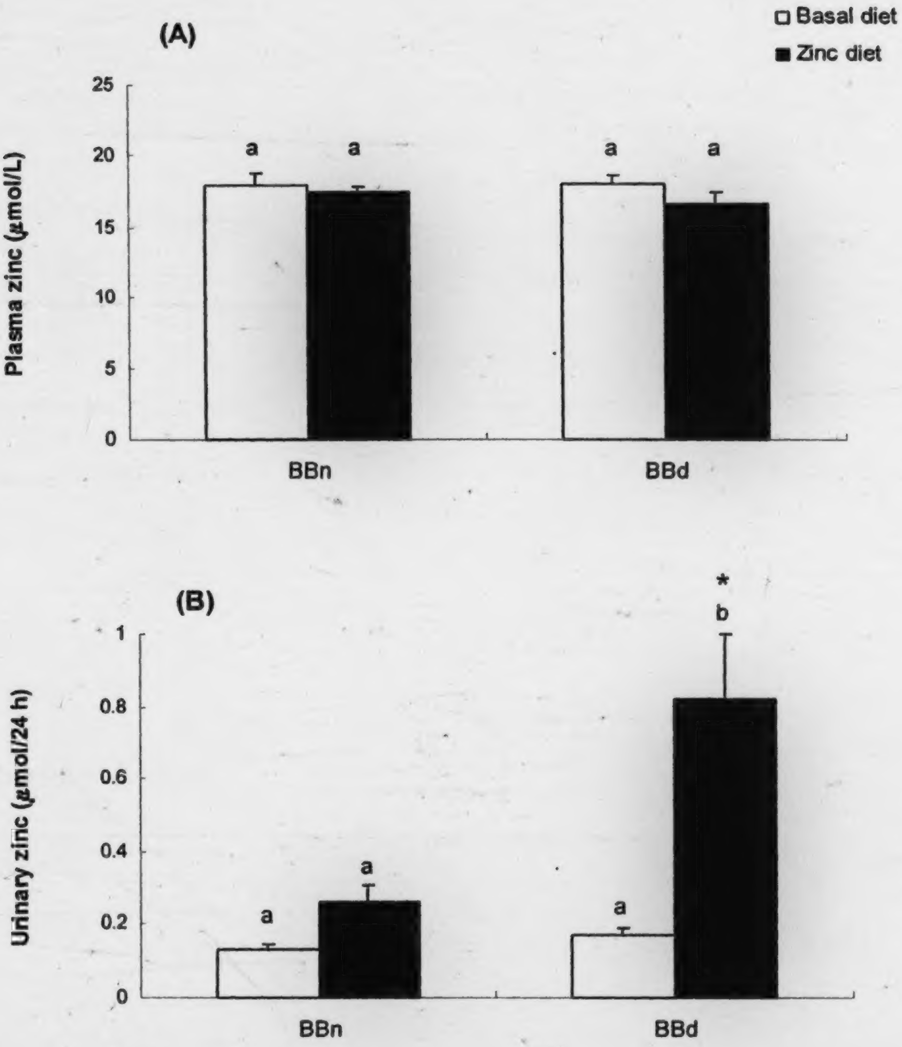

Figure 5-1 Plasma zinc levels (A) and urinary zinc excretion (B) in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

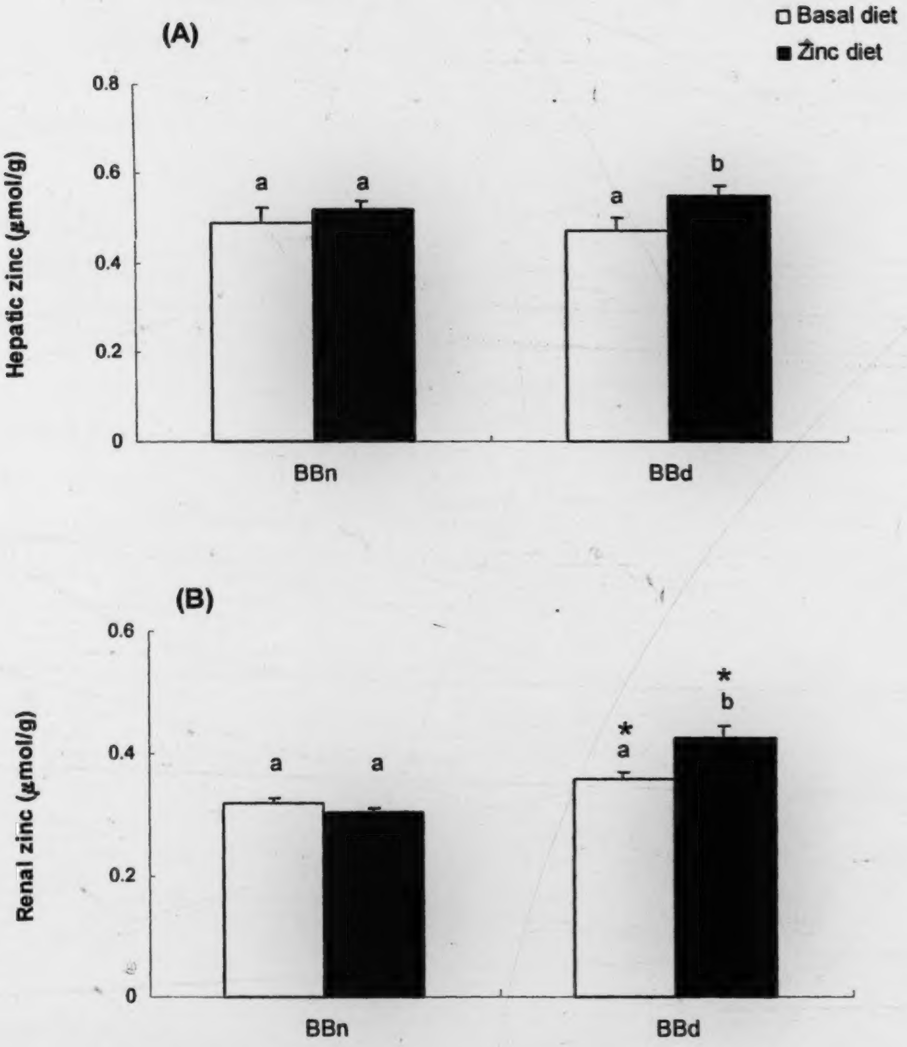

Figure 5-2 Hepatic (A) and renal (B) zinc levels in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

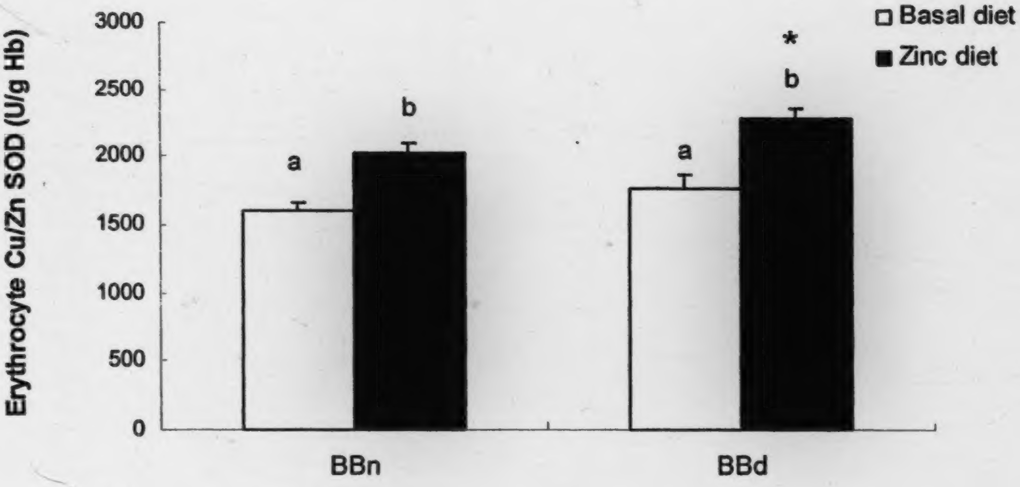

Figure 5-3 Erythrocyte SOD activities in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

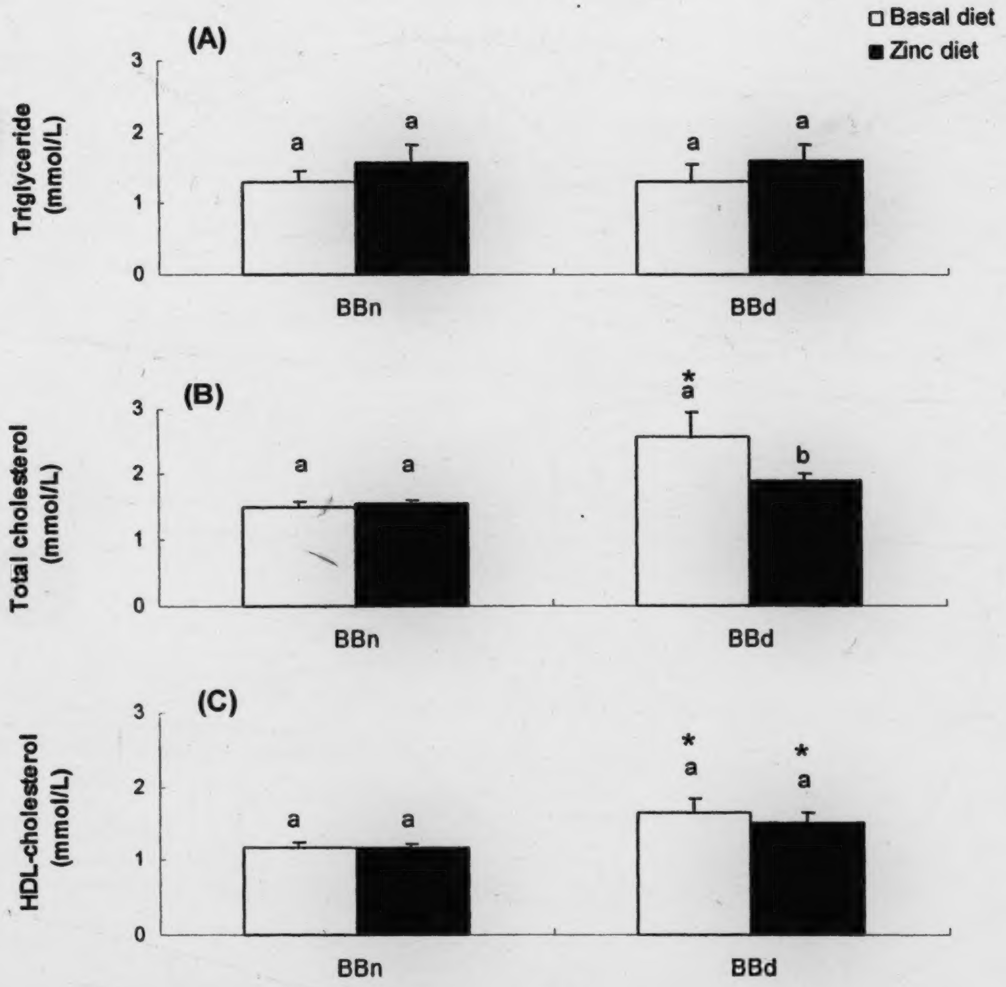

Figure 5-4 Plasma triglyceride (A), total cholesterol (B) and HDL-cholesterol (C) levels in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

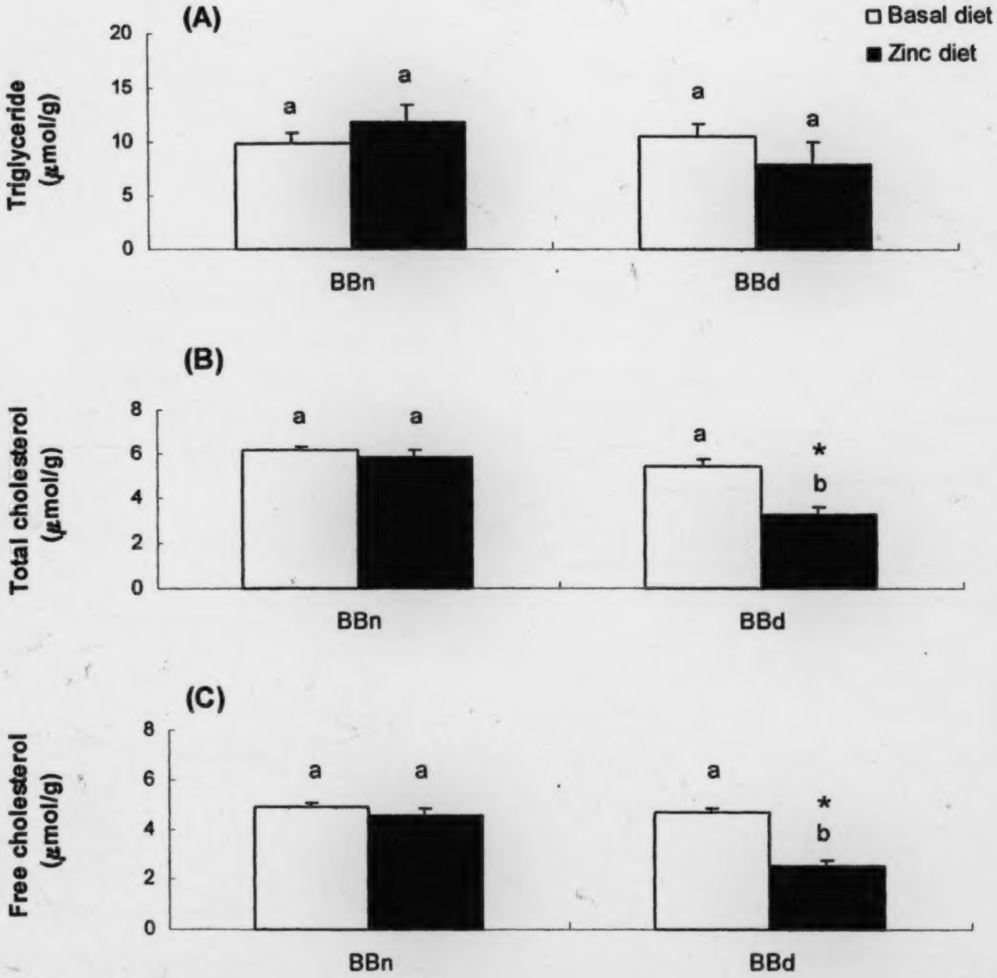

Figure 5-5 Hepatic triglyceride (A), total cholesterol (B) and free cholesterol (C) levels in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different (*P*<0.05). *(*P*<0.05) indicates a significant difference between the BBd and BBn rats fed the same diet.

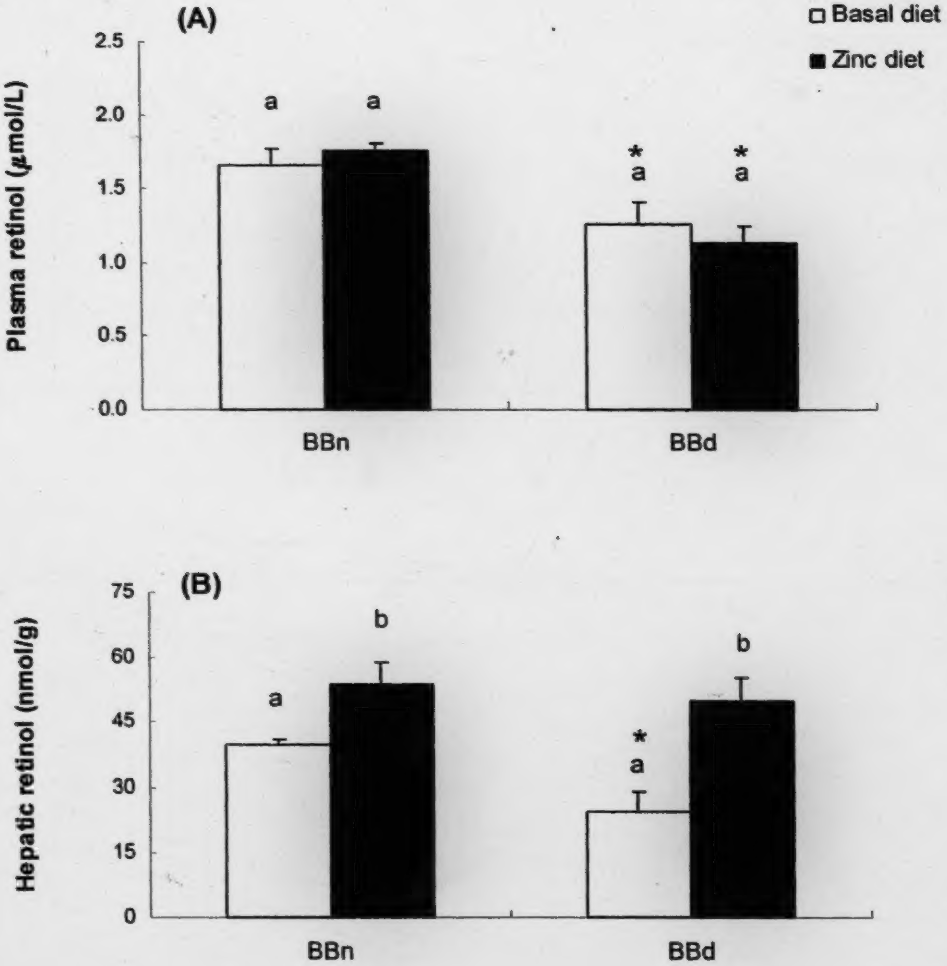

Figure 5-6 Plasma (A) and hepatic (B) retinol levels in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

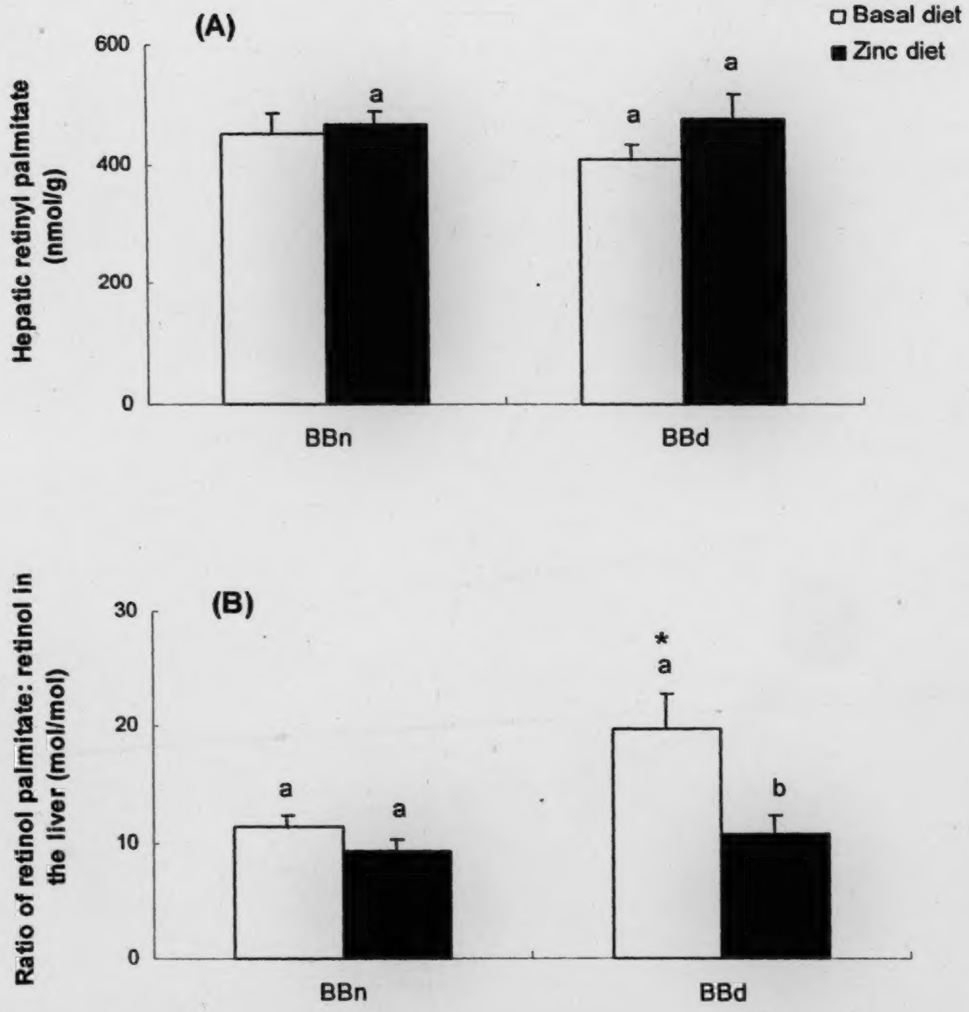

Figure 5-7 Hepatic retinyl palmitate levels (A) and the molar ratios of retinyl palmitate to retinol (B) in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

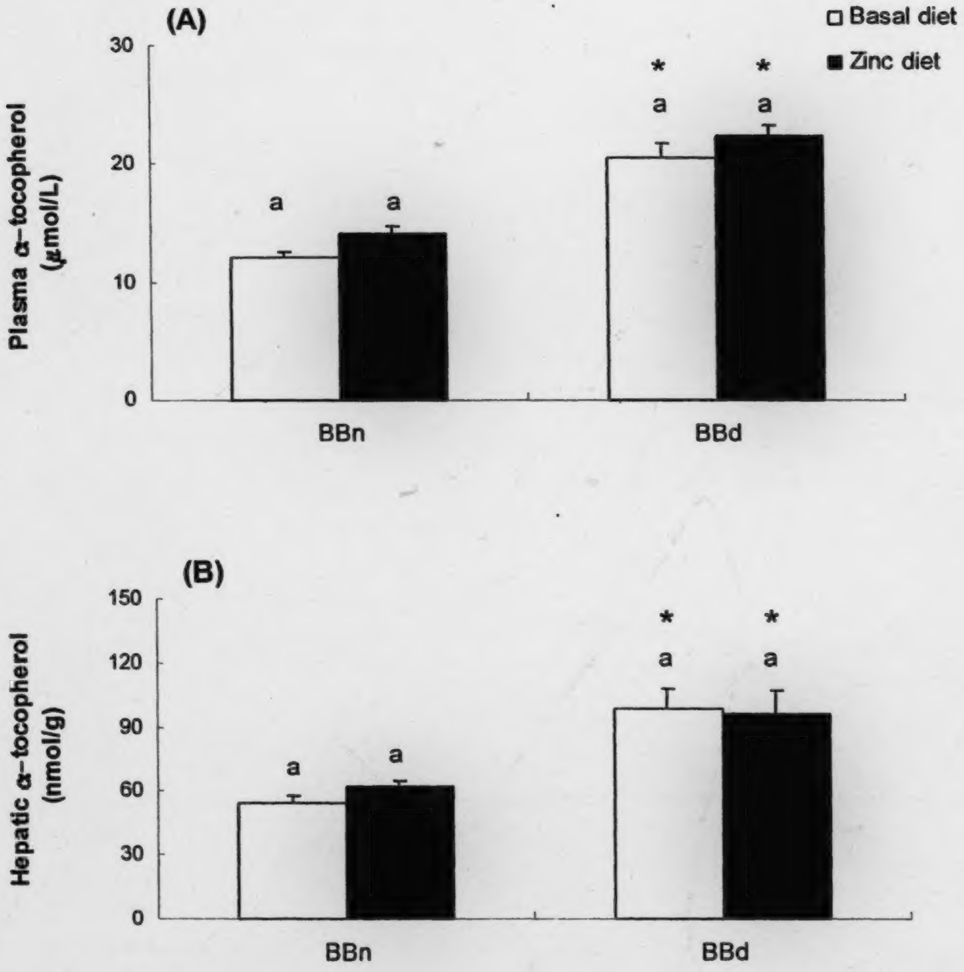

Figure 5-8 Plasma (A) and hepatic (B) α-tocopherol levels in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

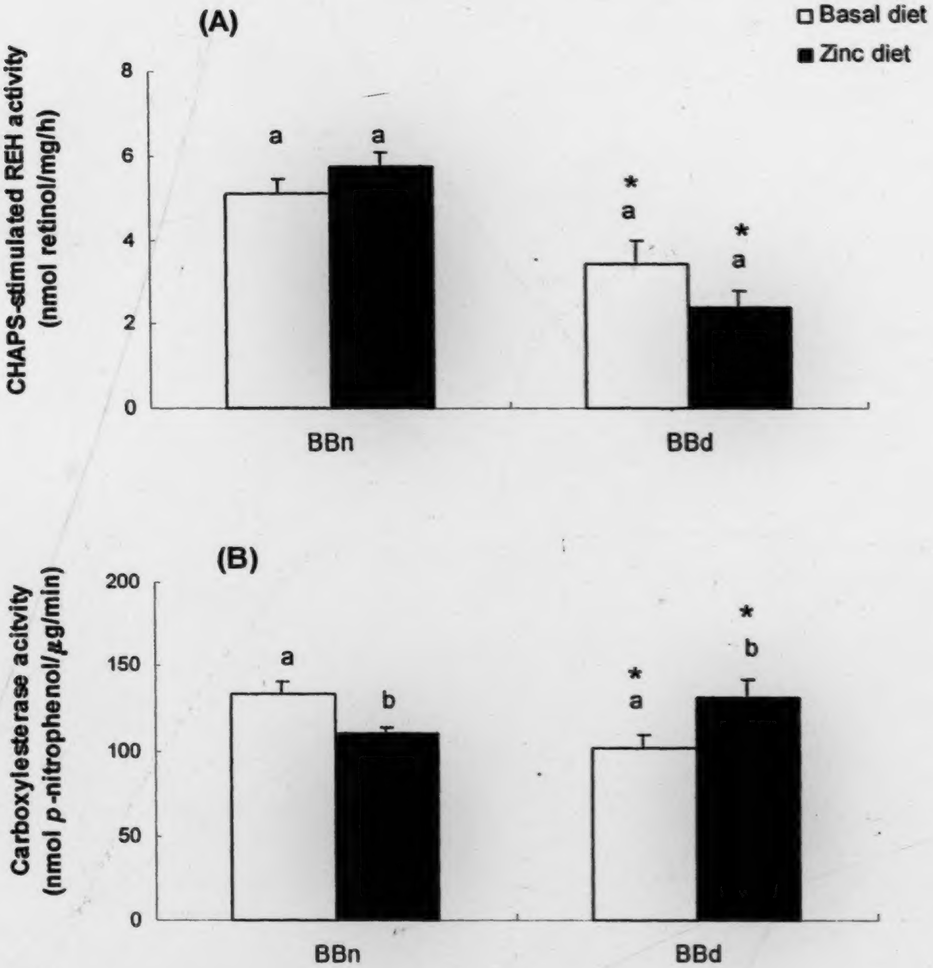

Figure 5-9 Hepatic CHAPS-stimulated REH (A) and non-specific carboxylesterase (B) activities in BBd and BBn rats fed a zinc diet or a basal diet.

The zinc diet (zinc-supplemented diet) contained 360 mg zinc/kg diet. The basal diet (NIH-07 diet) contained 60 mg zinc/kg diet. Data from rats fed a NIH-07 basal diet (see chapter 3) were used as data for controls. Values shown are means of seven or eight rats with their standard errors shown by vertical bars. For both BBn and BBd rats, bars that do not share a common letter within the same strain of rats are significantly different ($P<0.05$). *($P<0.05$) indicates a significant difference between the BBd and BBn rats fed the same diet.

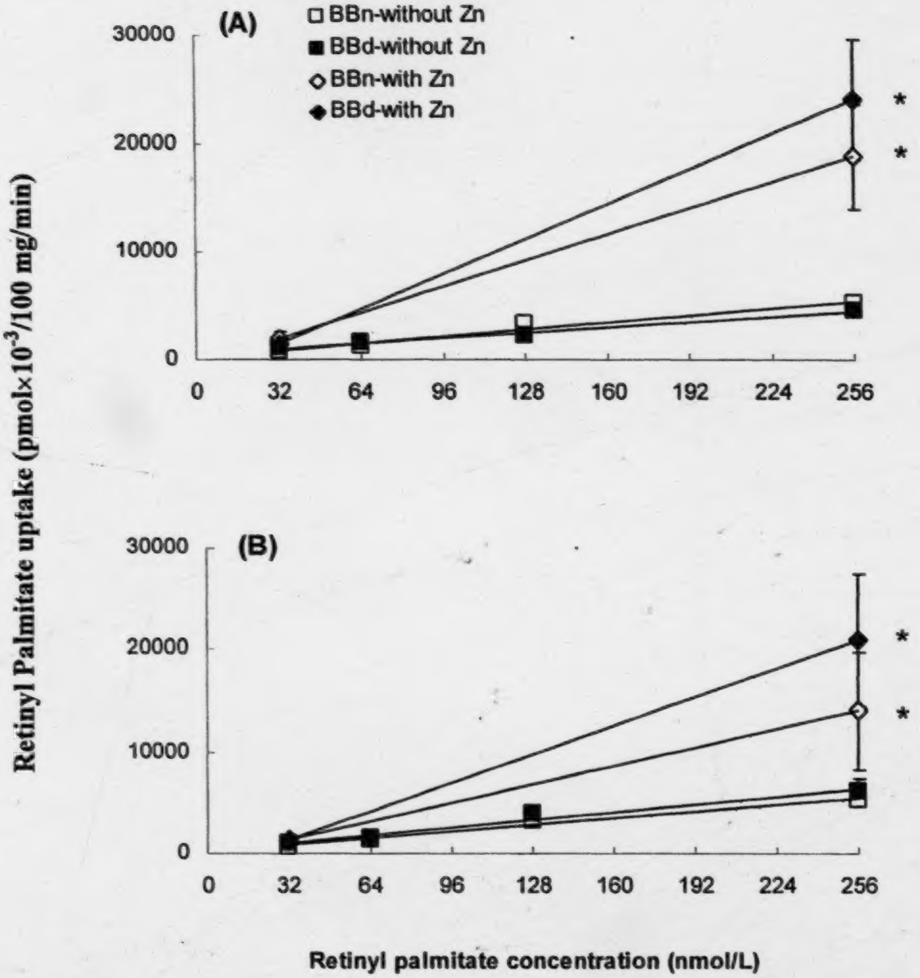

Figure 5-10 The uptake of retinyl palmitate into the jejunum (A) and the ileum (B) at different concentrations of retinyl palmitate without or with the co-existence of zinc *in vitro* in BBd and BBn rats.

Rats were fed a NIH-07 basal diet. Values are means for six rats with their standard errors shown by vertical bars. *($P<0.05$) indicates significant difference in the rate of retinyl palmitate uptake without and with zinc (0.153 mmol/L) in either BBd or BBn rats. No significant effect of diabetes on intestinal uptake of retinyl palmitate was detected irrespective of the existence of zinc in the incubation.

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Chapter 6

General discussion

The retinal pigment epithelial cell contains 95% of vitamin A as retinyl ester (Bridges 1976;Bok 1990). One of the important functions of this cell type is to provide rods and cones with a supply of 11-cis retinal, which is the key to the synthesis of rhodopsin and iodopsin, the visual pigments. A constant source of vitamin A must be available to maintain adequate levels of rhodopsin and optimal rod function. Low circulatory retinol level has been associated with an increased risk of epithelial damage in the eye and may ultimately lead to blindness (Thurnham 1997). In this study, the diabetes-prone BB rats displayed reduced plasma retinol levels before and at the onset of diabetes, suggesting that there may exist a genetically inherent abnormality in vitamin A metabolism in BB rats, a widely used animal model for type 1 diabetes in humans. Long before developing diabetes, BB rats have also been reported to have a reduced capacity to release insulin in response to glucose stimulation (Markholst and Lernmark 1988;Svenningsen et al. 1986). The decreased insulin response to glucose is parallel with a decreased content of this hormone in the pancreas (Markholst and Lernmark 1988). According to both *in vitro* and *in vivo* studies, vitamin A appears to be an important factor affecting insulin secretion (Chertow et al. 1987;Driscoll et al. 1997). Insulin release is reduced in vitamin A deficient rats and its secretion is restored to the normal level in rats repleted with retinyl palmitate. However, the cause and effect relationship between the biochemical evidence of vitamin A deficiency and the decreased insulin secretion in diabetes-prone BB rats is yet to be determined.

The reduced level of plasma retinol found in BB rats is not likely to be related to a low dietary intake of vitamin A because both diabetic rats and their controls consumed similar amounts of food during the experiment. The final body weight between these rats did not differ either. Besides, the hepatic total vitamin A and retinyl palmitate concentrations were not affected by the presence of diabetes. These results suggest that neither the intestinal absorption nor the hepatic uptake of vitamin A are of major concerns in the reduced plasma retinol level found in diabetic BB rats.

According to many studies, type 1 diabetes is associated with impaired metabolic availability of vitamin A, as evidenced by decreased plasma RBP and TTR levels as well as the hepatic RBP gene expression in the presence of diabetes (Tuitoek et al. 1996;Lu et al. 2000;Basu et al. 1989).

The importance of RBP in the mobilization of retinol from its hepatic stores has been supported by an earlier study, which found that RBP gene knockout mice had very low serum retinol levels while hepatic retinyl esters were accumulated in the liver of the mice (Quadro et al. 1999). Significantly decreased plasma retinol and RBP levels were also observed in TTR gene knockout mice although the hepatic retinol and retinyl ester levels in the mice remained normal (Wei et al. 1995). These studies suggest that the alteration of retinol carrier proteins may play an important role in causing the decreased plasma retinol level in a diabetic state.

This study is the first to demonstrate that, in addition to the retinyl carrier proteins, hepatic retinyl ester hydrolysis is affected in diabetic BB rats. This is evidenced by markedly decreased enzyme activities of CHAPS-stimulated REH, bile salt-independent REH as well as non-specific carboxylesterase in the liver of diabetic rats. Consistent with the alteration of REH activities in the liver, the molar ratio of hepatic retinyl palmitate to retinol in these rats was significantly elevated, suggesting that less retinol is liberated from hepatic retinyl ester by retinyl ester hydrolysis in the presence of diabetes. We also found that the activity of REH enzyme varies among different tissues in diabetic animals. Only the retinyl ester hydrolysis in the liver, but not in the intestine, was altered in diabetic rats. The activities of CHAPS-stimulated REH, bile salt-independent REH and non-specific carboxylesterase in jejunum and ileum were not affected in BBd rats compared with BBn controls. In agreement with this, we found no alteration on the *in vitro* uptake of retinyl palmitate into either jejunum or ileum in the presence of diabetes.

The underlying mechanism for the depressed hepatic REH activity in the presence of diabetes is not clear. It has been reported that the activities of some lipases including lipoprotein lipase and triglyceride lipase in tissues such as adipose tissue, muscle, liver and nerve were significantly reduced by low insulin level, and these enzyme activities were restored by insulin repletion (Duan and Sternby 1993;Blanco-Dolado et al. 2002;Ferreira et al. 2002;Nakai et al. 1979). REHs are a group of lipase functioning in retinyl ester hydrolysis (Harrison 1998). It is thus reasonable to suggest that REH activity might also be regulated by insulin level in a diabetic state. On the other hand, a markedly elevated vitamin E concentration, as shown in the present study, could contribute to the depressed REH activity as reported in other *in vitro* studies (Prystowsky et al. 1981;Napoli et al. 1984). Decreased REH activity in the liver may also be induced by a low protein diet (Tsin et al. 1986). However this is not the case in the present study, in which both diabetic and control rats were given a nutrient-complete diet throughout the experiments.

In this study, BB rats were fed diets containing vitamin A ranging from a borderline level (0.81 mg RE /kg diet), adequate level (2.61 mg RE/kg diet), to a high level (15.61 mg RE/kg diet) for an average of 65 d (from weaning until the onset of diabetes). The dietary interventions failed to alter the diabetes-associated reduction in plasma and hepatic free retinol concentrations in the rats. However, increased hepatic concentrations of total vitamin A were observed in response to a high vitamin A intake. As expected, neither the CHAPS-stimulated REH activity nor the non-specific carboxylersterase activity in the livers of these animals was altered by different dietary vitamin A intake.

This response of hepatic REH activity to dietary vitamin A appears to be different compared to another hepatic vitamin A metabolism-related enzyme LRAT. It has been reported that LRAT is at an almost undetectable level in vitamin A deficient rats but was increased after vitamin A supplementation (Dawson et al. 2000;Zolfaghari and Ross 2000). The different responses of REH and LRAT to dietary vitamin A intake may be explained by the two opposing reactions that the enzymes are involved in. REH hydrolyzes retinyl ester to liberate free retinol, which is then bound with RBP and transferred into the circulation. LRAT esterifies retinol to form retinyl ester, which is stored in the liver. Hence, it is likely that the capacity of hepatic retinol esterification, as indicated by LRAT activity, is regulated by dietary vitamin A intake as a homeostatic mechanism. The retinol is preserved for other essential processes (such as retinol-RBP complex formation and the synthesis of bioactive retinoic acid) during vitamin A deficiency, and is stored in the liver as retinyl ester when vitamin A is sufficient in the diet. On the contrary, REH activity seems to be maintained at a certain level despite the fluctuation of dietary vitamin A intake and hepatic vitamin A store. That ensures the availability of liberated retinol, and thus helps to maintain the plasma retinol level homeostatically.

It is reasonable to assume that hepatic REH activity might be altered under extreme circumstances such as severe vitamin A deficiency when all the stored retinyl esters are completely exhausted, or extra-large vitamin A intake when plasma retinol level can no longer be maintained homeostatically. However, neither of these circumstances was manipulated in the present study. In agreement with our results, a previous study found that vitamin A supplementation, at a level similar to that of our study, failed to increase plasma RBP and the free retinol levels back to normal in diabetic rats (Lu et al. 2000). It is thus concluded that vitamin A supplementation at the current level would neither improve hepatic REH activity nor increase plasma RBP concentration and therefore has no beneficial effect on the depressed hepatic vitamin A metabolic availability in diabetic rats.

It should be noted that, although we did not observe any sign of vitamin A toxicity in rats fed the high vitamin A diet, a significant amount of vitamin A was already accumulated in the liver of these rats. The vitamin A content in the high vitamin A diet in this study was only moderate (15.61 mg RE/kg diet) compared to other animal studies, in which rats were often fed diets supplemented with higher amounts of vitamin A (25 to 50 mg RE per kilogram diet) (Dawson et al. 1999;Dawson et al. 2000;Niu et al. 1987). Moreover, the average age of the rats in the present study was about 3 months, corresponding to adolescent humans. It is thus possible that a longer period of vitamin A supplementation, even at the current moderate level, may increase the hepatic accumulation of this vitamin and thus enhance the risk of vitamin A toxicity in the long run.

It was reported more than a decade ago that nearly half the elderly people in US routinely took nutrient supplements, which often included supplementation of vitamin A to a level that sometimes reached 5- to 10-fold above the Recommended Dietary Allowance (Hartz et al. 1988). With the rapid growth of the neutraceutical industry, we can expect that the consumption of nutrient supplements in the general population as well as in diabetic patients will continue to increase. It is known that vitamin A supplementation in large doses markedly increases plasma triglyceride and LDL-cholesterol level and decreases HDL-cholesterol concentration in humans and animals (Solomon and Erdman, Jr. 1980;Infante et al. 1991;Pastorino et al. 1991;Murray et al. 1983). These adverse effects of vitamin A supplementation on plasma lipids have been linked to an increased cardiovascular mortality in healthy people (Omenn et al. 1996). Similarly, we observed a significantly increased plasma triglyceride level and decreased HDL-cholesterol concentration in diabetic rats fed a high vitamin A diet. It is therefore possible that vitamin A supplementation might contribute to an increased risk of cardiovascular disease and other complications in diabetic population.

A significant interrelationship between zinc and vitamin A has been shown in a variety of clinical conditions such as liver cirrhosis, cancer and cystic fibrosis (Navarro and Desquilbet 1981;Herlong et al. 1981;Kapil et al. 2003). Zinc is necessary for the hepatic synthesis of RBP and CRBP, the former facilitates retinol transport from the liver to the circulation and eventually to target tissues, whereas the latter participates in retinol transport within cells (Smith et al. 1974;Kelleher and Lönnerdal 2001). Zinc deficiency has thus been linked to a decreased plasma retinol level despite adequate vitamin A intake, and zinc supplementation improves vitamin A status in both humans and animals (Smith, Jr. et al. 1976;Muñoz et al. 2000;Ette et al. 1979). Disturbed zinc status has commonly been reported to occur in the presence of diabetes as evidenced by increased urinary zinc excretions and decreased plasma zinc levels (Garg et al. 1994;Isbir et al. 1994;Brun et al. 1988). Altered zinc status was suggested to be partly responsible for the decreased plasma retinol levels in diabetic rats by reducing hepatic synthesis of RBP (Lu et al. 2000).

Results from our study further shows that dietary zinc supplementation significantly modified hepatic vitamin A metabolism in diabetic rats. That is evidenced by markedly increased hepatic free retinol levels and significantly reduced molar ratios of hepatic retinyl palmitate to retinol in the animals. These results suggest a shift of hepatic vitamin A from its storage to its free form occurs. In agreement with these observations, our study also found that the activity of hepatic non-specific carboxylesterase (one of the REH enzymes in the liver) was markedly increased with zinc supplementation in diabetic rats. However, the activity of another REH enzyme, CHAPS-stimulated REH, did not respond to an increased dietary zinc intake and remained at a low level in diabetic rats. These results are in agreement with others, who have reported that CHAPS-stimulated REH activity in the liver was not altered by zinc deficiency in rats (Boron et al. 1988). It is therefore possible that zinc participates in the regulation of some REH activities in the liver. On the basis of the findings reported here, it is reasonable to suggest that zinc supplementation may enhance retinol liberation from its hepatic storage pools through the regulation of REH activity and therefore increase the hepatic retinol availability in diabetic state.

Retinyl ester hydrolysis is also an essential step in intestinal absorption of dietary vitamin A (as retinyl ester). A 10-d zinc supplementation at a pharmacological dose level has been reported to significantly increase the pancreatic and intestinal hydrolase activities including lipase in rats (Szabo et al. 2004). Consistent with that result, our study found that zinc at a physiological level markedly increased the *in vitro* uptake rate of retinyl palmitate into either the jejunum or the ileum in both diabetic and normal BB rats. These results imply that similar to its influence on hepatic REH activity, zinc may potentially up-regulate intestinal REH activity and thereby modify vitamin A absorption in the intestine.

Although intestinal vitamin A absorption *in vitro* was markedly increased in the presence of zinc, we did not observe any change in the hepatic vitamin A store in rats supplemented with dietary zinc. This is probably because while zinc promotes the vitamin A absorption in the intestine, zinc may also enhance the hydrolysis of retinyl palmitate in the liver and thus liberate more retinol from its hepatic store. The resulting retinol is bound with RBP and is transferred to the circulation, which is regulated homeostatically in the body. Alternatively, the retinol may be degraded to form vitamin A metabolites such as retinal and retinoic acid due to the action of alcohol dehydrogenase and retinal oxidase before eventually being secreted into the bile (Hicks et al. 1984). Indeed, previous study has reported that the degradation of vitamin A in the liver is regulated by the zinc status (Boron et al. 1988). Zinc supplementation also increases bile acid secretion (Cho et al. 1985). Thus it is possible that while more vitamin A is absorbed in the intestine with zinc supplementation, more vitamin A in the liver is degraded to its metabolites and secreted into the bile.

In this study, we observed markedly elevated plasma total cholesterol and α-tocopherol levels in both prediabetic and diabetic BB rats. These changes may be linked to the alteration of insulin secretion since the latter plays a central role in the regulation of lipid metabolisms, such as promoting triglyceride clearance in the plasma (via lipoprotein lipase activation), inhibiting hepatic VLDL production and enhancing LDL clearance and degradation (Verges 1999). Insulin deficiency is often linked to increased triglyceride, LDL-cholesterol levels and decreased HDL-cholesterol concentrations in type 1 diabetes (Best and O'Neal 2000). It was not unexpected to see elevated plasma α-tocopherol levels along with increased total cholesterol concentrations in our study based on the fact that vitamin E shares a similar metabolic pathway with lipids. The absorption, transport and distribution of both vitamin E and lipids are very closely related (Traber and Sies 1996;Herrera and Barbas 2001).

Decreased HDL-cholesterol level has been reported in either zinc deficiency or in subjects supplemented with a large dose of zinc (Koo and Williams 1981;Schneeman et al. 1986;Koo and Lee 1989;Yousef et al. 2002;Umoren 1989;Cho et al. 1989;Black et al. 1988). This raised a concern about zinc supplementation in the human population. Our study demonstrated that zinc supplementation significantly decreased plasma and hepatic total cholesterol levels without altering plasma triglyceride and HDL-cholesterol level in diabetic rats. This favorable effect of zinc on lipid disorders in diabetes is supported by another study in which lower plasma levels of triglyceride and total cholesterol were found in diabetic rats receiving zinc supplementation (Wu et al. 2004). Zinc supplementation was also found to reduce lipid peroxidation in type 1 diabetic patients (Faure et al. 1995). These results further suggest that moderate zinc intake might be beneficial to diabetic subjects.

It appears that zinc supplementation in diabetic rats could increase hepatic retinol availability and RBP synthesis, as evidenced by increased hepatic free retinol levels and REH activity observed in our study. Lu's study also found that hepatic RBP gene expression was elevated in diabetic BB rats simultaneously supplemented with zinc and vitamin A (2000). To our disappointment, however, zinc supplementation failed to normalize the depressed plasma retinol level in the presence of diabetes despite a markedly increased hepatic free retinol level in diabetic rats. This lack of response of plasma retinol level to zinc supplementation was also observed by Lu et al (2000). It should be pointed out that BBdp rats were given a zinc-supplemented diet from weaning until 1 or 2 d after diabetic onset in both studies. Whether a longer period of zinc supplementation in rats in a diabetic state would help to improve their depressed plasma retinol level needs to be further explored.

In conclusion, zinc supplementation in type 1 diabetes is potentially warranted based on its positive influence on hepatic vitamin A metabolic availability. Vitamin A supplementation shows no beneficial effect on depressed vitamin A status in the presence of diabetes. However, an increased risk of vitamin A toxicity and cardiovascular disease may result in the long run. With the increasing use of nutrient supplementations in different populations, our findings may provide some fundamental evidence for better nutritional management of diabetes.

In the future, more work needs to be done to further investigate the contributory factors of depressed hepatic REH activity in type 1 diabetes. A challenging project would be to study the influence of CRBP on REH activity in the liver. Evidence has shown that this protein stimulates liberation of retinol from stored retinyl esters *in vitro* and that the apo-/holo-CRBP ratio might determine the rate of hepatic vitamin A hydrolysis through REH activities (Ghyselinck et al. 1999;Boerman and Napoli 1991).

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